Final Report for the DEFENSE LOGISTICS AGENCY (DLA)

STUDY OF THE EFFECT OF HYDROCARBON TYPE BIODEGRADATION ON FUEL SPECIFICATION PROPERTIES

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1. EXECUTIVE SUMMARY

The USAF is promoting initiatives such as the AF Smart Operations for the 21st Century (AFSO21) to maximize value and minimize waste. At the same time, the 2014 Energy Action Plan (2014 EAP) describes that the majority of the energy requirements for USAF operation is aviation fuel and that we need to assure its supply, reduce risks and improve resiliency by being prepared for multiple disruption scenarios; fuel biodeterioration may be one of those scenarios. In accordance to AFSO21, the AF Petroleum Agency and the Defense Logistics Agency have approved the reduction of fuel icing inhibitor (FSII) diethylene glycol monomethyl ether (DiEGME) in JP-8 fuel from 0.10 – 0.15% to 0.07 – 0.10% and they are in the process of moving to commercial Jet A (with additives). In the past, the transition from more toxic fuels and additives (JP-4 and ethylene glycol monomethyl ether - EGME) to less toxic ones (JP-8 and DiEGME) contributed to increased biodeterioration incidents. Reduction of FSII could further exacerbate fuel biodeterioration. More importantly, FSII doesn't protect against microbial growth happening at the fuel phase and headspace of the tank where fuel probes are typically placed. Tests performed in our laboratory showed that biodeterioration of materials in the fuel phase can occur even when the fuel itself appeared clean. Multiple field cases have indicated the occurrence of biodegradation of jet fuel filters and jet fuel bladder materials. Some costly problems associated with microbial growth include tank corrosion, fuel pump failures, filter plugging, injector fouling, topcoat peeling, and engine damage. Additionally, military ground vehicles, ships and power generators that use diesel fuel are very susceptible to biodegradation and biocorrosion issues due to the lack of FSII and the higher level of impurities in diesel which may enhance microbial growth.

Multiple studies had characterized the ability of microbes to adapt and thrive in fuel systems, creating contamination and biofilms in fuel storage tanks, pipelines, and aircraft wing tanks. JP-8, Jet A-1, Jet A, and F-76, are kerosene or diesel distillate products that can serve as a food/energy source to microorganisms. Previous testing has demonstrated that bacteria, including the ubiquitous fuel degrader *Pseudomonas aeruginosa*, show a preference for specific hydrocarbon types, which could slightly change fuel composition, leading to possible changes in fuel properties.

In order to investigate if microbial contamination can potentially change the bulk or trace properties of fuels, experiments were conducted with several kinds of fuel-degrading microbes including *Pseudomonas aeruginosa* (freshwater bacteria), *Acinetobacter venetianus* (freshwater bacteria), *Marinobacter hydrocarbonoclasticus* (seawater bacteria) *and Yarrowia lipolytica* (Yeast/fungi); these studies were performed in small laboratory scale fuel reactors. In these experiments the fuels were exposed to microbial growth for periods of time that ranged from two weeks to 90 days, and may have included the use of a pure microorganism or a , mixture of two microorganisms, to determine if synergistic or competing effects were observed. In most of these experiments, sealed containers were used to decrease the amount of evaporation of fuel volatile components, but these containers were regularly vented to replace the oxygen used up by the microbes in the sealed vessels. Experiments were conducted in containers as small as 8 mL, using 10 µL of fuel, and as large as a 50 L container, using 1 liter of fuel. Analytical testing, using GC, GC-MS and GCxGC instrumentation was conducted on the fuel to determine how the fuel was changing chemically, as well as on the aqueous portion of the mixture. Growth was tracked spectrophotometrically over the incubation periods to determine if sufficient growth

was being achieved. Specification testing was conducted with the larger samples of fuel to examine whether specification tests were altered by microbial exposure.

Over the previous 12 months, we investigated whether microbes could consume enough fuel components to change the properties of the fuel. Because certain bacteria can target specific compounds within the fuel, and the composition of jet fuel is directly related to its physical and chemical properties, it was prudent to determine whether fuel properties could be affected in this way. We observed that microorganisms were able to degrade significant amounts of *n*-alkanes (Figures 1-7); this degradation accounted for a 4 to 6% reduction in the total fuel n-alkane content (Tables 11, 16, 17, 20, 21, 26). This large reduction in normal alkanes resulted in changes in low temperature properties related to freeze point and pour point which was lowered in every case between 4 and 7 °C (Tables 7, 10, 13 and 15). Some microbes such as *Marinobacter* were able to efficiently degrade some aromatics in fuel including ethylbezene, ethylmethyl benzene and toluene but this degradation was not enough to significantly change the total aromatic content of the fuel (Figures 3 and 4). In most cases, the total aromatic concentration in the fuel appeared to increase by about ~2%; this increment was not enough to bring the aromatic content of the fuel above the maximum limit allowed in the fuel tested. The observed increase in aromatics per volume was likely a function of the decrease in the total normal alkanes (Figures 8 and 9). However, for fuels close to aromatic specification limits, a decrease in n-alkanes might cause an increase in aromatic content which could make the fuel off-specification in aromatics. Results of other fuel bulk property tests showed no changes or very small changes. As an example, the acid number of F76 and Jet A appeared marginally changed (Tables 10 and 15) but not enough to be of concern.

However, one area that showed dramatic changes was the formation of emulsions (foam) and biofilms. When fuel was exposed to the bacteria *Acinetobacter* we observed that a large portion of the fuel was emulsified into a gel-like foam that was nearly impossible to dissolve in both non-polar and polar solvents (Figure 24). We demonstrated that this emulsified fuel (foam) was able to plugged filters preventing normal fuel flow (Figure 33). This results correlate with the publish literature which presents that material entrained in a flow system will create significant fuel system problems including filter plugging (as measured by pressure drop), poor fuel flow and disarming of coalescers.

Another factor affecting fuel specification is the presence of additives added to fuel. Additives are added to fuel to prevent corrosion, fuel oxidation, fuel icing, biodegradation, and to improve lubricity. Degradation of these additives by microbes may lead to long-term problems. The additives examined were a corrosion inhibitor/lubricity improver (CI/LI), the fuel system icing inhibitor diethylene glycol monomethylether (DiEGME) also known as FSII, and a fuel antioxidant. Antioxidants are not required additives, but are often added at the refinery to slow oxidation in storage or under thermal stress conditions. Corrosion inhibitors are required in many fuels because they interact with metal surfaces to slow corrosion. More importantly, the corrosion inhibitors also provide fuel lubricity enhancement which is important when fuels are highly hydroprocessed. Hydrotreating removes the natural lubricants in fuel, and this removal may cause fuel pump failures and increase wear and tear of moving parts over prolong periods or under extreme conditions. The active ingredient in many CI/LI is fatty acids such as di-linoleic acid. Bacteria, yeast and fungi can actively degrade fatty acids so we expected that CI/LI may be highly degraded. The most common antioxidant for fuel, butylated hydroxytoluene (BHT) was

also studied here. Our experiments showed that exposing fuel with BHT to bacteria did not lead to the degradation of BHT (Table 29). A possible explanation for this is that BHT is: 1) highly non-polar, which prevented it being solubilized into the water phase where microbes reside, and 2) recalcitrant to degradation due to the toxic nature of its aromatic ring and side chains.

However, when we tested the degradation of the CI/LI additive Unicor-J by exposing fuel containing CI/LI to the fuel-degrading yeast *Yarrowia,* we found that 78% of the CI/LI was consumed by the microorganism (Table 31). Previous work has shown that lubricity, as measured by the Ball-on-Cylinder Lubricity Evaluation (BOCLE) is directly related to corrosion inhibitor concentration. This microorganism was able to grow to high cell densities in the presence of 20 mg/L CI/LI additives (Figure 31), but we have not yet confirmed corresponding degradation of lubricity by the BOCLE test. This result presents important implications for fuel lubricity and corrosion prevention and requires further study.

We have also shown that even the fuel system icing inhibitor (FSII) DiEGME which is considered a biocide can be degraded by microorganisms. DiEGME is added to fuel at a concentration of 0.07 – 0.10% (v/v) and then can partition and accumulate in the water phase to high concentration (30-60% v/v). We have tested whether DiEGME at low concentration, such as those added to fuel, can be degraded by bacteria. The results indicated that the bacteria *Pseudomonas* was able to degrade between 60% and 68% of the DiEGME added at concentrations between 0.05 and 0.2% (v/v) in water (Table 32). Further, the bacteria was able to degrade 30% of the DiEGME even when this was added at ten times (1% v/v) the maximum concentration currently allowed in fuel (Figure 32). We observed that DiEGME was first

converted into a short-lived intermediate compound which was finally converted into a stable oxygenated metabolite (Table 32). The amount of this oxygenated analyte was proportional to the amount of DiEGME being consumed.

Finally, we showed that it is crucial for DLA, the AF and DoD to further investigate the effects of biodiesel in fuel even when this biofuel might be present at trace levels. Currently, commercial aviation is considering increasing the allowable level of biodiesel in jet fuel from 5ppm to 100ppm, with possibility of allowing higher levels in the future. Here we have shown that biodiesel in diesel blends dramatically increased microbial growth, biofilm formation and emulsification (Figure 33). Further, we have observed that even trace levels (ppm) of biodiesel in jet fuel can exacerbate microbial growth (Figure 34) and the formation of organic acids products of FAMEs degradatation (Figure 35). These organic acids may contribute to other detrimental effects including biocorrosion.

The results of this study indicate that it may be difficult for microbes to change the fuel bulk properties that are directly associated with the fuel hydrocarbon composition because it would require the production of very large biomass, much larger than what can be commonly attained in a real fuel tank with small amounts of water. However, our results clearly show that other factors in the fuel including additives (CI/LI, FSII, others) and trace levels of FAMEs may affected how microbes behave and grow, and could eventually adversely affect fuel bulk properties.

The changes in lubricity that occur when fuel is exposed to bacteria requires further detailed study. In Table 34, we show that control samples improve in lubricity, possibly due to exposure to ionic nutrients in the growth media. However, in Table 32 we also showed that CI/LI is significantly reduced because of consumption by (at least) some microbes, notably *Yarrowia*. We know therefore, that media exposure and consumption by microbes are opposing forces with respect to changing fuel lubricity. A more thorough understanding of these forces, as well as a more complete understanding of the various CI/LI compounds would be an important research area that should be addressed in the future.

From the results the following conclusions are made:

1) microbes can degrade specific hydrocarbons in fuel in any system, depending on factors such as type of microbe, availability of minerals, availability of water, etc., but that bulk properties (density, distillation range, aromatic content, etc.) are not affected greatly.

2) there is a potential to disrupt fuel properties and performance as demonstrated by specification tests that are related to, or controlled by, fuel additives (corrosion, lubricity) and trace impurities

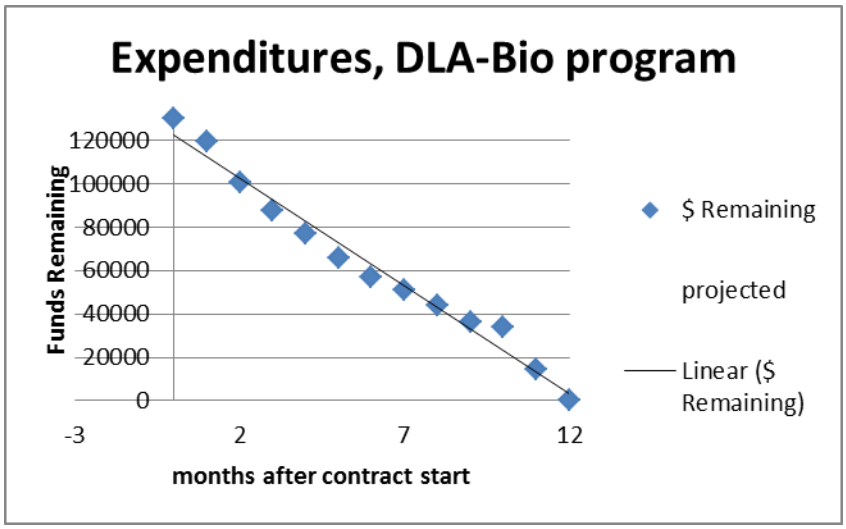
3) biofilms and fuel emulsions (foams) that are formed by the microbes are more immediate and visible changes than fuel compositional changes, and these biofilms do create operational problems;

4) additive concentrations can be affected by microbial degradation. However, it is possible that these changes may be negligible depending on the fuel storage conditions since additives are replaced when a tank is refilled with fuel.

5) trace contamination of jet fuel with fatty acid methyl esters or FAMEs (biodiesel) and low molecular weight alcohols such as ethanol can may exacerbate fuel biodeterioration and formation of biofilms; this appears to be an immediate problem that should be addressed in detail given the desire to increase FAME contamination specifications in jet fuel and the current use of multiple diesel-biodiesel blends.

Financial Summary

Month	Monthly Labor	Labor Hours	Monthly M&S	Total Expenditures	$ Remaining
					$130,000
June 2013	$10,141	109	$ 300	$10,141	$119,859
July 2013	$18,756	238	$ 0	$18,756	$101,103
August 2013	$11,082	134	$2,343	$13,425	$90,021
September 2013	$ 10,187	111	$ 0	$10,187	$ 79,834
October 2013	$ 9,273	114	$1,971	$11,244	$ 65,947
November 2013	$ 8,850	96	0	$8,850	$ 57,097
December 2013	$ 6,204	69	0	$6,204	$ 51,193
January 2014	$ 7,354	58	0	$7,354	$ 43,839
February 2014	$ 7,259	68	459	$7,718	$ 36,121
March 2014	$ 2,517	24.5	0	$2,517	$33,604
April 2014	$18,958	239	0	$18,958	$14,646
May 2014	$ 14,373	198	273.02	$14,646	0


Expenditures, DLA-Bio program
Funds Remaining
120000
100000
80000
60000
40000
20000
0
-3
2
7
12
months after contract start
$ Remaining
projected
Linear ($ Remaining)

2. INTRODUCTION

Biological growth and its effect on the cleanliness of aviation, marine and ground fuels is a significant concern to both military and civilian fuel users. Water can be introduced into fuel tanks, aircraft or ground equipment by condensation or through leaky infrastructure; moisture can often collect at the bottom of tanks exacerbating microbial growth and biodegradation. Bacteria are ubiquitous in the environment but with fuel and water available, bacteria have the necessary minerals and carbon source to thrive. Extensive growth and biofilm formation can lead to costly and disruptive problems in fuel systems including tank corrosion, fuel pump failures, filter plugging, injector fouling, topcoat peeling, and engine damage (Edmonds and Cooney, 1967; Passman et al., 2001, 2012; Jung et al., 2002; Rauch et al., 2006; Brown et al., 2010; Korenblum et al., 2010; White et al., 2011). Besides these real effects, there has been speculation about the effects of microbes on the actual fuel composition and quality. For example, it has been reported that microbes can severely reduce the concentration of lubricity improvers present in diesel fuels, increasing wear in pumps and at other high friction surfaces (Stamper et al., 2012). Because kerosene and diesel distillate products may serve as food and energy source to microorganisms, it is possible that rampant growth could lead to measureable changes in fuel composition.

Multiple bacterial species have been isolated from environments exposed to fuel (Edmonds and Cooney, 1967; White et al., 2011) with *Pseudomonas aeruginosa* and *Marinobacter hydrocarbonoclasticus* being ubiquitous hydrocarbon degraders of terrestrial and marine environments, respectively (Belhaj et al., 2002; Hua et al., 2006). Bacteria and other microorganisms in fuel tanks prefer to live in the water/fuel interface, feeding on the hydrocarbons, thereby having a nearly limitless supply of fuel available to grow (Saadoun, 2002;

Sanger and Finnarty, 1984). While the fuel is a source of energy for the bacteria, components of it may be toxic to the organism; many bacteria have evolved protection mechanisms such as the formation of biofilms and the activation of efflux pumps for protection (Gunasekera et al., 2013). Biofilms are collections of cells enclosed in a matrix of polymeric compounds, primarily exopolysaccharides (EPS) (Vu et al., 2009). Biofilm formation in fuel tanks and its importance have been described (Passman, 2003). The combination of biofilms and the cellular material from organisms can create significant masses that can disable filters, plug flow paths and generally upset the intricate nature of the fuel delivery system for aircraft and ground engines.

P. aeruginosa, a rod-shaped, gram-negative bacteria, is known to metabolize normal alkanes in fuels *via* oxidation and use them as the sole carbon source for growth. *P. aeruginosa* has two alkane hydroxylases (alkB1 and alkB2), cytochrome P450 and other essential electron transfer proteins that allow it to metabolize medium and long normal alkanes (Gunasekera et al., 2013). The *P. aeruginosa* bacteria were previously characterized (Striebich et al., 2014) to determine which hydrocarbon species from a complex mixture it preferred to consume. This specie showed that normal alkanes, primarily the longer chain alkanes, were preferred by this organism. Some *Pseudomonas* species have also been known to consume other hydrocarbons including aromatics and cyclic hydrocarbons but generally these hydrocarbons appear to be more toxic to microorganisms (Corwin and Anderson, 1967; Inoue and Horikoshi, 1989; Li et al., 1998). *Marinobacter hydrocarbonoclasticus* is another example of a rod-shaped, gram-negative aerobic bacteria recently isolated from marine environments polluted with hydrocarbons. While the exact pathways by which *Marinobacter* can metabolize multiple hydrocarbons are not understood, this bacteria is known to degrade normal alkanes, primarily the lower molecular weight alkanes, by the action of the alkane monooxygenases encoded by the *alk*B genes (Smith

et al., 2013). *Marinobacter* was also capable of consuming lighter, low molecular weight alkylbenzenes (Striebich, et al., 2014), but showed a tendency to prefer certain isomers over others.

These studies provided a general understanding about what compounds each microbe will consume while in a fuel environment. In spite of this understanding, ***this research program focuses on changes that may occur in the fuel, not just on the molecular level, but on a property measurement level.*** For example, whether or not bacteria will degrade a particular aromatic compound may be important for understanding or characterizing its behavior. The more important concern is whether the bacteria will affect a change in any of the specification property for the fuel. For example, if bacteria produced aromatic waste products from alkanes, the percent aromatics specification test would be affected. Bacteria may produce waste products at ppb to ppm levels that may be important for the overall properties of the fuel. But it is critical to understand if this 1 ppm contamination will lead to failure of specification tests for properties such as thermal stability, as measured by the Jet Fuel Thermal Oxidation Tester (JFTOT) or existent gum, as measured by ASTM D381. These two measurements, in particular, have been developed over the years to indicate when fuels might be expected to cause performance problems in aircraft such as filter plugging, injector fouling, heat exchanger surface deposition and the like.

However, fuel specification tests can be difficult to perform for experiments where fuel is exposed to bacterial degradation. The specification tests themselves, for Jet A in particular, can require about 2 gallons of fuel to fully characterize performance properties. In order to expose that much fuel to bacteria in a reasonable amount of time, large tanks for aqueous media and bacteria are required. In order to conduct this research in a laboratory in a reasonable timeframe,

we chose to conduct experiments initially with small amounts of water and fuel and examine specification properties by non-specification methods. These methods are generally newer, and require less fuel than the existing, approved methods. In this research, we conduct experiments exposing fuel, first in small amounts, measuring both chemical composition and specification properties with small amounts of fuel, and then increase the size of the experiment in order to attempt to use the actual specification test methods to determine whether fuel is affected.

At the conclusion of these tests, we hoped to understand how fuel changes when it is exposed to bacteria, both on a molecular level and on a fuel specification level. We hoped to understand if bacterial contamination will affect fuel properties, and what properties are expected to change. Our approach to the bacterial contamination has been to have as small an amount of fuel as possible, increasing the chance that we will be able to see the degradation, but large enough volumes of fuel that select specification tests can be conducted.

3. EXPERIMENTAL

3.1 Types of experiments conducted and fuel:media ratios

In this effort, we have been setting up exposures of microbes to jet fuels, diesel fuels and other hydrocarbons. In performing these exposures, we were very careful to control the ratio of fuel to media (fuel:media) because the analytical techniques (GC, GC-MS and GCxGC) used cannot distinguish the small changes that are produced by microbes when the fuel volume is too large in comparison with the aqueous (water) phase. Therefore, in these experiments, we used a 1:10 fuel:media ratio or lower (such as 1:50 or 1:100). Higher ratios (such as 1:5 or 1:1) are more difficult to see reaction, because the small changes bacteria create are not observable if there is an excessive amount of fuel. Generally, screening studies were conducted in 8mL vials with 1

mL of media and 10 µL of fuel (1:100 fuel:media ratio). Intermediate-sized experiments were performed in 50 mL conical vials, where a 10 mL media sample were mixed with a 100 µL or 1 mL fuel volume (1:100 or 1:10 fuel:media ratio, respectively). These intermediate-sized experiments (1:10 ratios) were generally conducted for longer periods of time than the vial experiments (1:100 ratios). For larger volumes of fuel, needed to conduct specification testing, a 1 L Teflon bottle containing 100 mL of media and 10 mL of fuel were used. By combining multiple Teflon bottles with 100 mL of media and 10 mL of fuel, a reasonable size sample was collected in order to conduct select specification tests. These types of experiments had to be conducted for longer periods of time (up to 28 days or longer) in order to get measureable results. For larger volumes than this, bioreactors of either glass or plastic were used. The largest bioreactor used in this project was a 50 L polypropylene bag, holding 10 L of media and 1 L of fuel. These experiments were typically conducted for even longer periods of time, with some experiments conducted for three months. Table 1 provides a summary of the types of containers and volumes of fuel and media used for the majority of the experiments conducted.

3.2 Individual Biological Exposures Conducted

In this effort, there were hundreds of exposures of various fuels to bacteria in several different containers for different durations. Initially, 8 mL glass vial experiments were conducted which served to help us understand, from a chemical composition point of view, which compounds would be consumed by certain bacteria or fungi, and under what conditions this might happen. These initial experiments were important to guide larger scale, longer duration experiments and were also conducted throughout the study in order to understand a particular experiment or series of experiments.

Other than the 8 mL vials, used to conduct screening experiments for better understanding of microbial behavior, many other longer term studies were conducted and are listed in Table 2. Some of these experiments helped to optimize conditions or uncovered some experimental problems and will not be discussed in the results section of this report. For example, *Acinetobacter* experiments were conducted in "DLA-08" showed some serious evaporation problems, which were caused by autoclaving plastic materials to the point where lids did not seal. Results of positiveexperiments are the main topic of the results sections.

Table 1. Sample containers and fuel:media ratios for experiments to investigate changes in Specification Tests.

Sample container	Manufacturer, part number	Fuel volume	Media volume	Headspace volume	Sealed or unsealed
8 mL Glass vials, plastic lids	Fisher Scientific, 03-340-60B	10 µL	1 mL	7 mL	sealed
Plastic, 50 mL conical vials	Nunc Polypropylene tubes, ThermoScientific 339653	1 mL	10 mL	39 mL	sealed
Teflon, 1 L bottles, lids	Cole Parmer	10 mL	100mL	890 mL	Sealed, but regularly vented
2 gallon Carboy	Thermo Scientific	300 mL	3 Liters		Sealed, but occasionally vented
50 L polypropylene bag	ThermoScientific, HyClone BioProcess Container, 50L	1 L	10 L	25 L	Sealed, gas withdrawn and re-introduced

Table 2. Experiments conducted for large scale DLA program to investigate fuel specification property deterioration due to microbial degradation of the fuel.

DLA experiment number	Microbe or fungus	fuel used	duration (days)	Fuel:media ratio	Container volume	harvested
0	various	various	various	1:100	8 mL	various
1	*Pseudomonas Aeruginosa*	**F76**, POSF-10448	40	1:10	1 L bottles	5/28/2013
2	*Pseudomonas Aeruginosa*	**F76** (POSF-10448) and **Jet A** (POSF-4658)	28	1:10	1 L bottles	7/18/2013
3	*Pseudomonas Aeruginosa*	**F76**, POSF-10448	70 days	1:10	50 L bag	9/12/2013
4	*Pseudomonas Aeruginosa*	**Jet A,** POSF-4658	91 days	1:10	2 gallon carboy	7/18/2013
5	*Pseudomonas Aeruginosa*	**F76,** POSF-10304 and **Jet A**, POSF-4658	28 days	1:10	1 L bottles	8/29/2013
6	*Marinobacter Hydrocarbono-clasticus*	Hydro-refined diesel (**HRD**), POSF-8374	28 days	1:10	1 L bottles	9/2/2013
7	*Yarrowia lipolytica*	**F76,** POSF-10304 and **Jet A**, POSF-4658	18 days	1:10	1 L bottles	9/25/2013
8*	*Acinetobacter venetianus*	**F76,** POSF-10304 and **Jet A**, POSF-4658	28 days	1:10	1 L bottles	9/25/2013
9	*Consortia: Pseudomonas aeruginosa and Acinetobacter venetianus*	**Jet A,** POSF-4658	28 days	1:10	1 L bottles	10/21/2013
10*	*Sequential: Pseudomonas aeruginosa then Acinetobacter venetianus*	**Jet A,** POSF-4658	14 days of *Ps . A .*, 14 days of *Ac. V .*	1:10	1 L bottles	10/24/2013
11*	*acinetobacter venetianus*	**Jet A**, POSF-4658	28 days	1:10	1 L bottles	10/25/2013
12	*acinetobacter venetianus*	**Jet A,** POSF-4658	27 days	1:10	1 L bottles	12/5/2013
13	*Sequential: Pseudomonas aeruginosa then Acinetobacter venetianus*	**Jet A,** POSF-4658	14 days of *Ps . A .*, 14 days of *Ac. V .*	1:10	1 L bottles	12/23/2013

* experimental results suspect due to evaporation or other fuel loss

3.3 Vial experiments

Bioassays conducted in vials were performed to understand the behavior of the microbes before conducting larger scale testing. The purpose of the vial experiments was to create an assay that could determine 1) whether or not the microbes in question could grow under the conditions tested; 2) quantify the growth using growth curves as measured at 600 nm; and 3) to determine what compounds within the mixture were being consumed while the microbes grew. While many organisms have been tested for growth in fuel, the microbes in Table 3 were chosen for larger scale testing due to their common frequency of occurrence in fuel systems and their behavior in fuel.

Table 3. Microbes used in vial testing which were also investigated using larger scale studies.

organism	fuel used	duration (days)	Fuel:media ratio	Container volume
Pseudomonas aeruginosa	**F76** (POSF-10448) and **Jet A** (POSF-4658)	14 days	1:100	8 mL
Marinobacter Hydrocarbonoclasticus	**HRD-5 (highly-refined diesel), Jet A** (POSF-4658)	14 days	1:100	8 mL
Acinetobacter venetianus	**F76** (POSF-10448) and **Jet A** (POSF-4658)	14 days	1:100	8 mL
Yarrowia lipolytica	**F76** (POSF-10448) and **Jet A** (POSF-4658)	14 days	1:100	8 mL

The vial bioassays were performed by using 10 µL of Jet-A, F-76 or some other fuel of interest in 990 µL of M-9 minimal media (90mM $Na_2HPO_4 \cdot 7H_2O$, 22 mM KH_2PO_4, 8.5mM NaCl, 18.6 mM NH_4Cl, 2mM $MgSO_4$, 0.1mM $CaCl_2$ and 0.02mM $FeSO_4$) containing one or more of the microorganisms listed in Table 3 at 0.03 optical density (OD_{600nm}) in a 8 mL glass vial sealed with a PTFE-lined lid. The lids were tightened and sealed with parafilm. The samples

were maintained in a 28 ^{o}C incubator for a period of between 10 to 14 days, without opening the vial. Multiple sample replicates were incubated at the same time, and then sample vials were removed from the incubator at the time of testing. Control samples were also prepared in separate vials containing the same proportions of fuel and minimal media but without bacteria. Each vial was used for a single test, including optical density measurement or measurement of the fuel components remaining in the sample by GC. The OD of the inoculated medium was measured using a SmartSpec Plus Spectrophotometer (Bio RAD).

The media mixtures were changed when a bacteria from a marine environment was being tested. Simulated seawater was added to the media to provide more nutrients for growth (Striebich, et al, 2014). Some bacteria were slow to grow and occasionally, vial experiment may have been conducted for longer periods of time than 14 days. Samples containing fuel were incubated for up to 21 days at 28 °C.

3.4 Sample preparation and GC analysis

During vial testing, samples of the fuel/M-9 mixture were analyzed using a liquid-liquid extraction with methylene chloride (Fisher Optima, purity, > 99.9%), followed by GC-MS or GCxGC analysis. Methylene chloride extraction of water, soils and other solids is a routine technique for isolating organics from aqueous or solid matrices (Burford et al., 1993). For each sample, an internal standard (Ultra Scientific) and 2.0 mL of methylene chloride were added to the M-9/fuel mixture and shaken by hand for one minute. The vials were then placed in an ultrasonic bath (10 minutes) to assist the methylene chloride extraction process and break emulsions within the sample. The samples were centrifuged (LK Industries); the methylene chloride, containing any organic components from the original 10 µL of fuel, or decomposition

products from the bacteria, was removed to a GC vial for analysis. Because different jet fuel components have different solubility in aqueous systems, this liquid-liquid extraction technique removed all the organics from the sample to be collected, whether in the organic phase or aqueous phase.

GCxGC (Johnson and Synovec, 2002; Striebich et al, 2011; Van der Westhuizen et al., 2011) was conducted using a non-polar 30 meter primary column (DB5-MS, Agilent Technologies) and a shorter, 5 meter secondary column (DB-17, Agilent Technologies), using hydrogen carrier gas; other analytical conditions are previously described (Striebich et al., 2011) and an example of a 2-dimensional separation for fuel is shown. GCxGC provides more resolution because there are two distinct separations occurring for the entire chromatogram. Another significant advantage of this system was that a flame ionization detector (FID) and a mass spectrometer (MS) measured chromatographic response simultaneously, providing the ability to perform qualitative and quantitative analysis of the samples.

In the larger bottle experiments, it was desirable not to solvent extract the fuel from the mixture, but rather to collect it. Generally, these experiments, which were conducted to determine the effect of microbial degradation on fuel specification test results, required that larger samples of fuel be collected. Therefore, GC, GC-MS, and GCxGC testing were conducted on the collected samples without extraction. Whether the samples were collected by extraction, or collected by simply sampling the fuel and then diluting, the same chemical analysis procedures were conducted.

3.5 Other analytical procedures and specification testing.

Normally, GC, GC-MS and GCxGC were conducted on most samples. However, when enough fuel was collected, specification testing was conducted. When possible, the actual specification test was conducted according to the ASTM procedure specified for that test. However, there was often insufficient fuel for the traditional specification tests, so other tests were used which required less fuel. In many cases, the substitute tests were more modern, requiring less fuel, and generally measuring the properties very accurately. The specification tests and non-specification tests used in these evaluations are listed in **Table 4** for F-76 and **Table 5** for Jet A and JP-8. Jet A was primarily used in this study given that the Air Force is transitioning from JP-8 to commercial Jet A as the main aircraft fuel. Defense Logistics Agency (DLA) purchases both fuels and therefore would be interested in growth of microbes in both fuels and subsequent degradation of specification properties. So while Jet A was used in testing, the results provide insight into what may happen when biological growth occurs in JP-8 which has similar bulk properties to those of Jet A.

Table 4 shows the specification tests appropriate for F-76, a diesel fuel formulation used by the US Navy. It differs from the Jet A or JP-8 specification in that there is no freeze point requirement, but there is a pour point and a cloud point requirement. There are distillation requirements too, although different in magnitude than the Jet A. There is no thermal stability testing in this particular specification.

All of the specification tests conducted was performed according to each of the ASTM methods appropriate for the test. In some cases, substitution of a modern test was used, in order

to save fuel. Low temperature properties such as freeze point, cloud point and pour point were performed using the PhaseTek instrument, which uses 0.75 mL to conduct the measurement of all three properties, instead of traditional methods. Another example of a substitution is the Quartz Crystal Microbalance test which is a research device to substitute for evaluating thermal stability, the property normally evaluated by the Jet Fuel Thermal Oxidation Tester (JFTOT). The JFTOT test requires 500 mL or more of fuel, and it is difficult to accumulate that much fuel significantly exposed to microbes.

Table 4. Specification Tests, Properties and Limits for F-76

Method	Test	Min	Max
ASTM D4176-04	Appearance		
ASTM D93-13	Flash Point, (°C)	60	
ASTM D5773-10	Cloud Point, (°C)		-1
ASTM D5949-10	Pour Point, (°C)		-6
ASTM D445-12	Viscosity at 40°C	1.7	4.3
ASTM D86-12	Distillation		
	10% Recovered , (°C)	report	
	50% Recovered , (°C)	report	
	90% Recovered , (°C)		357
	End Point , (°C)		385
	Residue, (°C)		3
ASTM D130-12	Copper Strip Corrosion (2 h at 100 C)		1 (max)
ASTM D4294-10	Total Sulfur (% mass)		0.05
ASTM 524-10	Carbon Residue, 10% Bottoms (% mass)		0.2
ASTM D482-13	Ash (mass %)		0.005
ASTM D976-06	Cetane Index, Calculated	43	
ASTM D974-12	Acid Number (mg KOH/g)		0.3
ASTM D7171-05	Hydrogen content by NMR, (% mass)		
ASTM D4052-11	Density @ 15 C (kg/L)		
ASTM D6045-12	Color, Saybolt		
ASTM D7688-11	HFRR Lubricity @60C, Wear Scar diam (mm)		

Table 5. Specification and non-specification tests and limits for JP-8 and Jet A used in evaluating the effect of microbes on fuel quality.

ASTM Test #	SPECIFICATION TESTS	JP-8 spec min	JP-8 spec max	Jet A spec min	Jet A spec max
	Workmanship		pass		pass
D3242	Total Acid Num. (mg KOH/g)		0.1		0.1
D1319	Aromatics, vol %		25		25
D3227	Mercaptan Sulfur, wt %		0.002		0.003
D4294,1266,1552,2622	Total Sulfur, wt%		0.3		0.3
D86 or D2887	Distillation D86 or D2887				
	IBP, deg C		Report		Report
	10% recovered, deg C		205		205
	20% recovered, deg C		Report		Report
	50% recovered, deg C		Report		Report
	90% recovered, deg C		Report		Report
	EF, deg C		300		300
	Residue, vol%		1.5		1.5
	loss, vol%		1.5		1.5
D93	Flash point, degrees C	38		38	
D5972	Freeze Point, degrees C		-47		-40
D445	Viscosity @ -20, cSt		8		8
D3338	Heat of Comb. (calc), BTU/lb	18400		18400	
D1322	Smoke Point, mm	19		18	
D1840	Naphthalenes, vol %		3		3
D130	Copper Strip Corrosion		1		1
D3241	Thermal Stability @ 260°C				
	Tube Deposit Rating		<3		<3
	Change in Pressure, mmHg		25		25
D381	Existent Gum, mg/100mL		7		7
D5452	Particulate Matter, mg/L		1		1
	Filtration Time, minutes		15		15
D1094	Water Reaction		1B		1B
D5006	FSII, vol%	0.1	0.15	none	none
D2624	Conductivity, pS/m	150	600	50	450
D4052	API Gravity @ 60 F	37	51	37	51
D4052	specific gravity, g/mL	0.84	0.775	0.84	0.775
	SPECIAL AND NON-SPECIFICATION TESTS				
D6379	Aromatics, vol % by HPLC		Report		Report
D445	Viscosity @ -40, cSt		Report		Report
D4809	Heat of Comb.(meas),BTU/lb	Report		Report	
D5865	net Heat of Comb. (meas), BTU/lb	Report		Report	
D3343	Hydrogen Content, wt %	13.4		13.4	
D5001	Lubricity (BOCLE), mm		Report		Report
Phase-Tek	Freeze Point, degrees C		-47		-40

4. RESULTS

4.1 Preliminary Vial Experiments

4.1.1 *Pseudomonas aeruginosa* degradation

Vials differ greatly from larger experiments in the sense that the available bacteria can consume a greater percentage of the available hydrocarbons, such that the hydrocarbons consumed can be distinguished more readily from those not-consumed. The measurement techniques (in these experiments) are mainly gas chromatography-mass spectrometry (GC-MS) and these techniques have a finite limit as to their sensitivity in comparative studies. If bacteria available can eat 5 nanograms of a certain compound during a 4 day experiment, and the experiment was initiated with 5 grams of the certain compound, it is most unlikely that a difference will be observable. However, if the experiment begins with 50 nanograms of the compound, and the bacteria consume the compound at the same rate (5 ng/4 days), a relatively short experiment of 4 days would show a 10% reduction in the amount of the compound available. The vial experiments concentrate on a very small amount of nutrient (fuel) in the vials, with 10 μL of jet fuel being the amount most often (almost exclusively) used. Table 3 shows the pertinent vial experiments conducted using Jet A fuel, F-76 fuel and various microbes. Figures 1 and 2 shows the result of these experiments, conducted in vials for *Pseudomonas aeruginosa*.

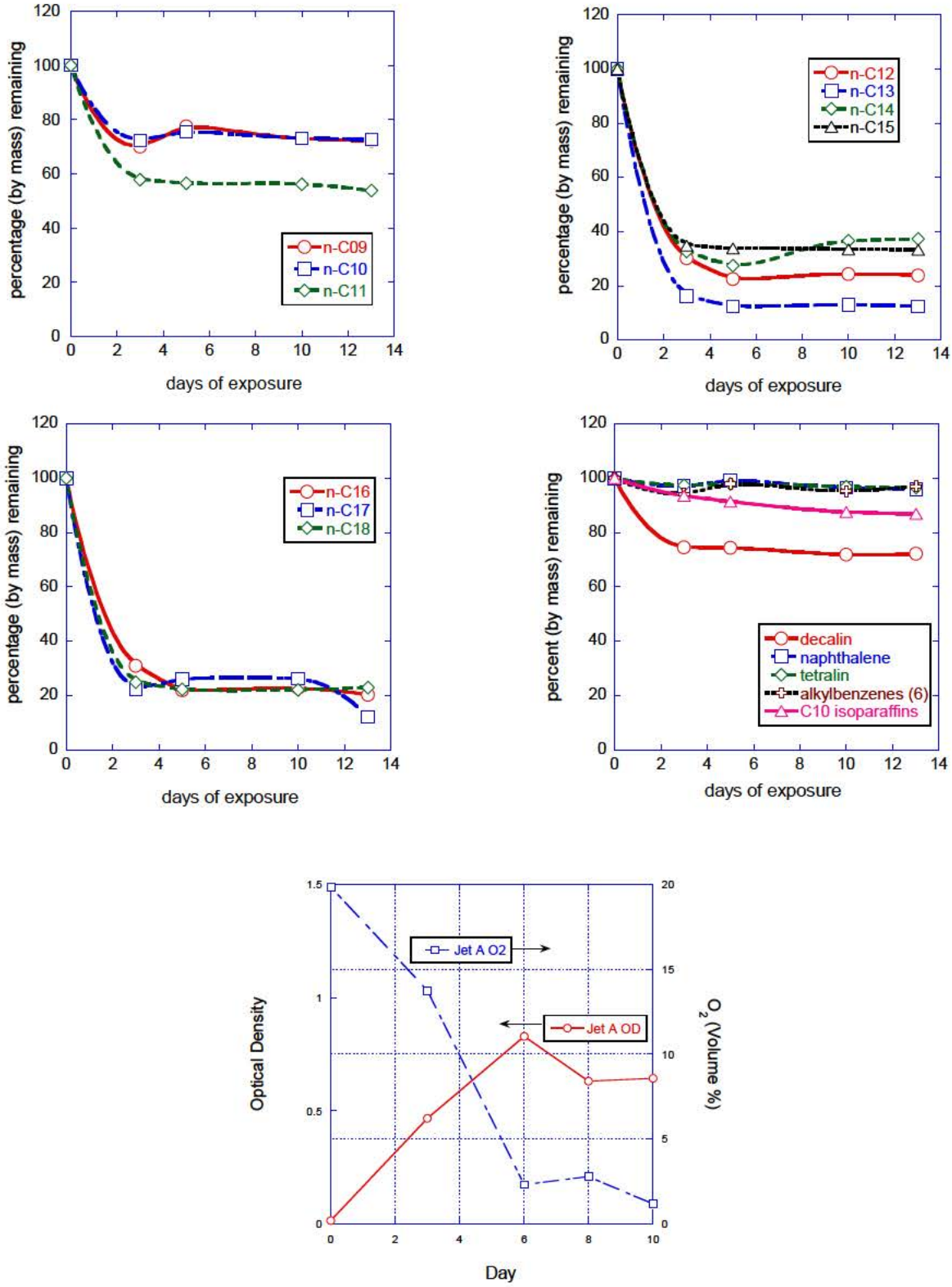


Figure 1. Thirteen day degradation of Jet A exposed to *Pseudomonas aeruginosa* in vial-sized bioassay, with a 1:100 fuel to media ratio. The bottom plot shows the growth curve for the Jet A exposure to *Pseudomonas*.

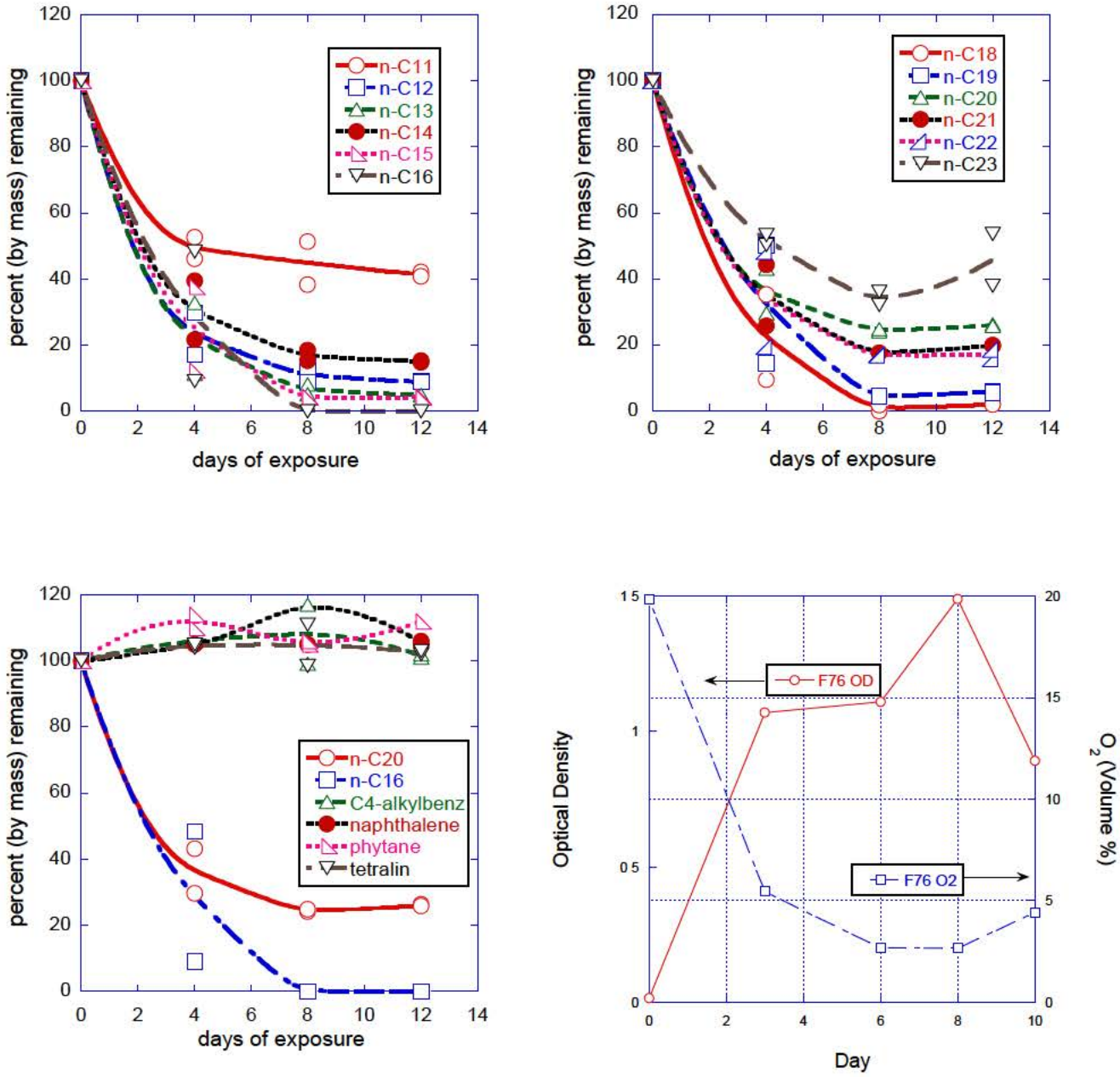


Figure 2. Thirteen day degradation of F76 exposed to *Pseudomonas aeruginosa* in vial-sized bioassay, with a 1:100 fuel to media ratio. The bottom right plot shows the growth curve for the F76 exposure to *Pseudomonas*.

It is clear from these data that the organism *Pseudomonas aeruginosa* is an excellent degrader of n-alkanes and more accurately, is an excellent degrader of the heavier n-alkanes of Jet A and F-76. In Figure 1, the C8, C9, C10 and C11 n-alkanes are not degraded as much as the heavier n-alkanes between C12 and C18 for Jet A. No other significant degradation of any other compound occurred with *Pseudomonas* (other than decalin). Similarly, F-76 degradation by *Pseudomonas* was characterized by n-alkane degradation between C12 and C22. There was no degradation of any aromatic compounds like naphthalene or alkylbenzenes, or even branched alkanes as shown. Thus, overall, we consider *Pseudomonas aeruginosa* to be an excellent degrader of n-alkanes, between C12 and C22, but not aromatics or isoparaffins.

4.1.2 *Marinobacter hydrocarbonoclasticus* degradation

Marinobacter was a slow-growing microbe which was a completely different hydrocarbon degrader than *Pseudomonas*. This microbe showed significant degradation of the C11 and lower normal alkanes in Jet A. It also readily degraded some of the C1, C2 and C3 substituted alkyl benzenes, but interestingly, not all were degraded (Figure 3). In F76, *Marinobacter* still shows a tendency to consume the light normal alkanes, and a select few of the straight chain substituted aromatic compounds (Figure 4). *Marinobacter* is fairly consistent in degrading similar compounds between the two fuels, in spike of the fact that the fuels have much different concentrations of light, volatile compounds due to their boiling ranges.

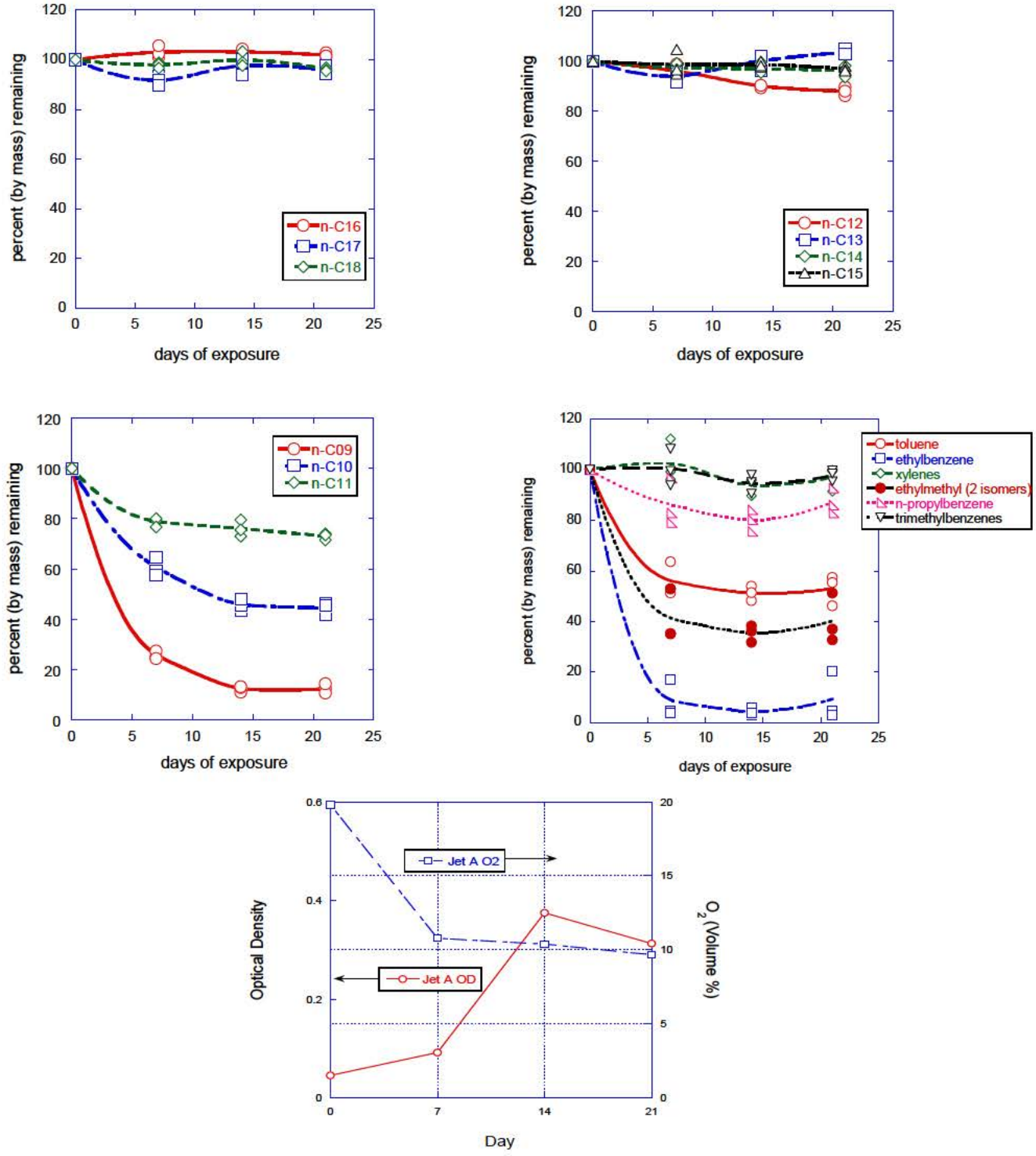


Figure 3. Twenty-one day degradation of Jet A exposed to *Marinobacter hydrocarbonoclasticus* in vial-sized bioassays, with a 1:100 fuel to media ratio. The bottom plot shows the growth curve for the Jet A exposure to *Marinobacter*.

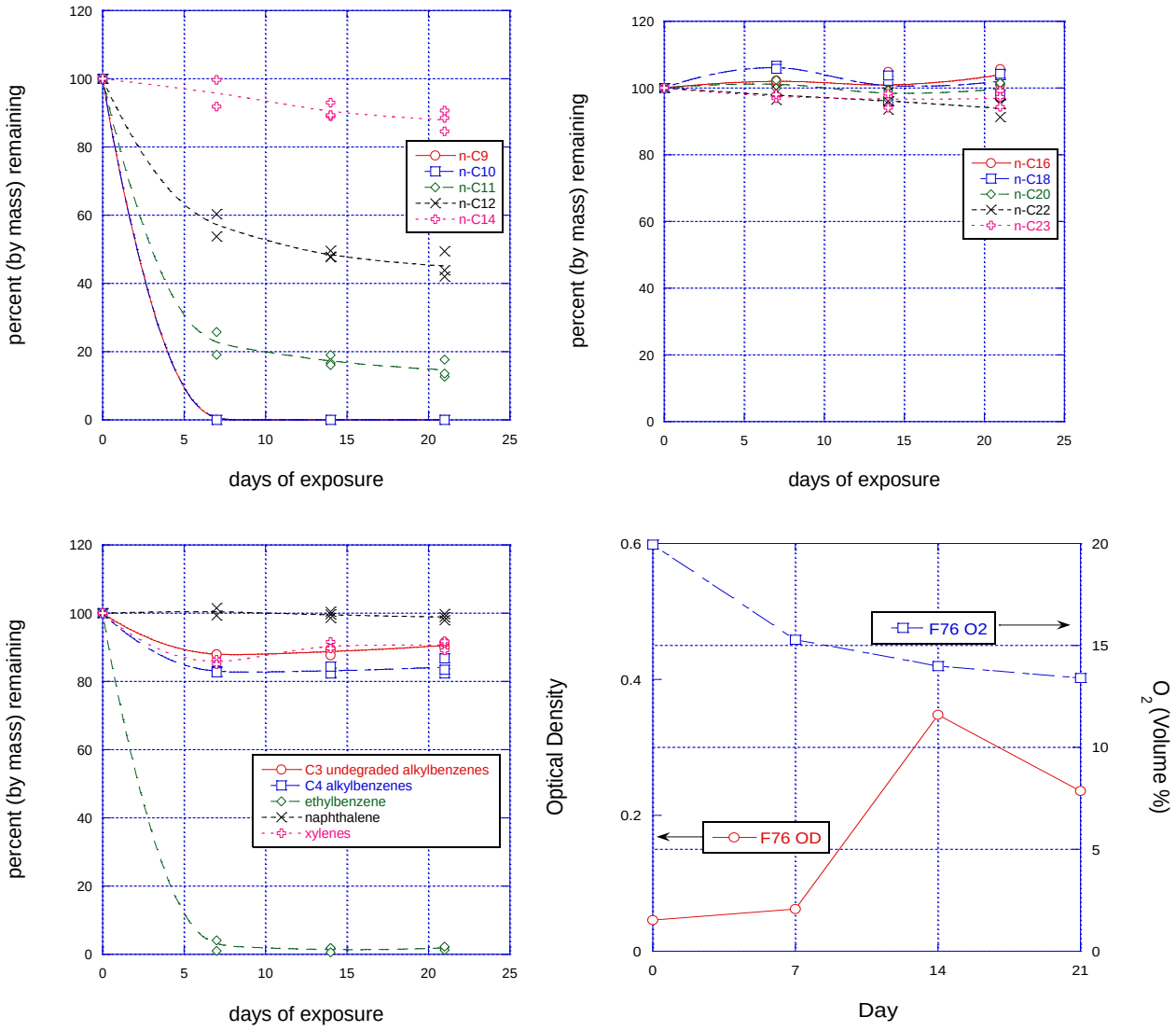


Figure 4. Twenty-one day degradation of F76 exposed to *Marinobacter hydrocarbonoclasticus* in vial-sized bioassays, with a 1:100 fuel to media ratio. The bottom right plot shows the growth curve for the F76 exposure to *Marinobacter*.

4.1.3 *Acinetobacter venetianus* degradation

As one of the most common contaminants of fuel systems, the bacteria *Acinetobacter venetianus* was tested in vials at 1:100 fuel to media ratios for 21 days. These bacteria were tested in Jet A, and the result from GC analysis is provided in Figure 5 below. This chromatographic comparison shows that almost all normal alkanes are reduced over a period of 21 days, as are some of the branched alkanes, but none of the aromatic compounds. The red line on the chromatogram shown in Figure 5 provides the level of the normal alkanes in the exposed sample. It is easy to see in this comparison that the height of each normal alkane has been reduced somewhere between 30-60% of the original amount. Also, some of the isoparaffins have also been reduced. This chromatogram was processed such that the individual components could be evaluated for their degradation levels over the course of the experiment (Figure 6). The general information about this bacteria is that it readily degrades normal alkanes over the entire Jet A range of hydrocarbons and that it does not significantly degrade alkylbenzenes, cycloparaffins, diaromatics or highly substituted branched alkanes. It does, as mentioned earlier, degrade some of the isoparaffins.

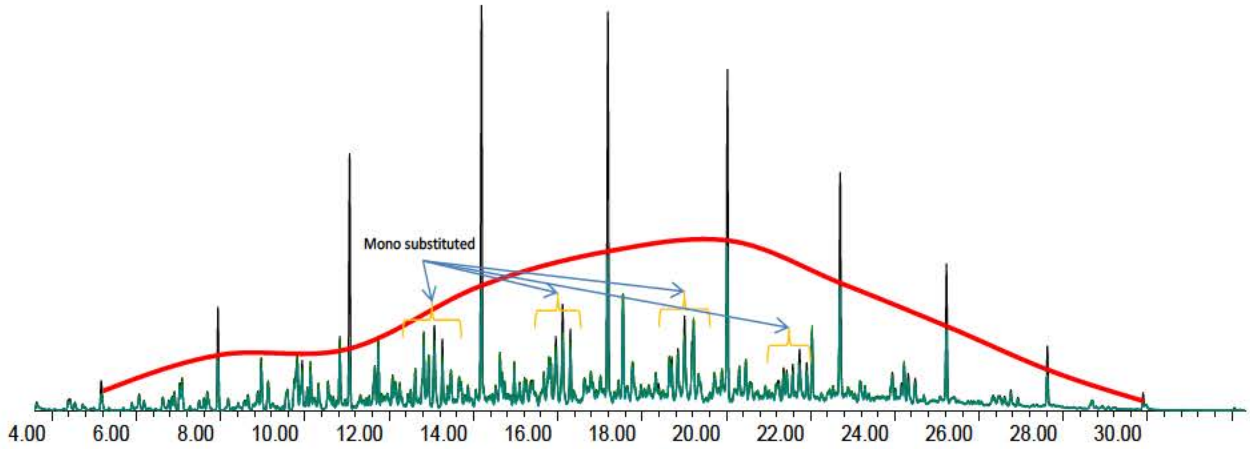


Figure 5. Microbial degradation of Jet A by *Acinetobacter venetianus*. Red line shows the level normal alkanes in the green chromatogram (day 21)versus the black (day 0) chromatogram.

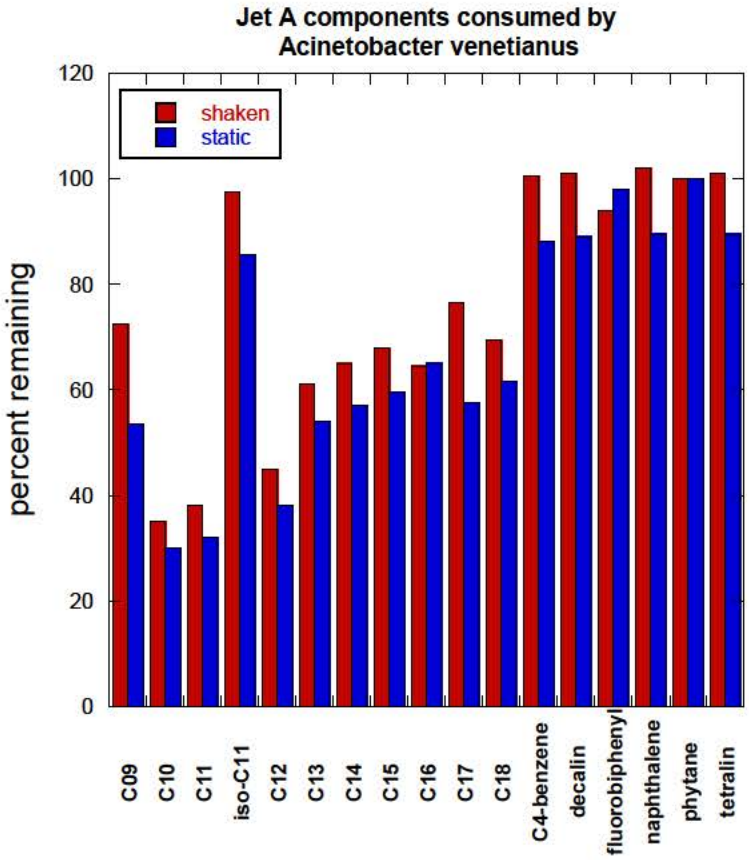


Figure 6. Bar chart showing comparison of two vial experiments with *Acinetobacter* showing the degradation of n-alkanes and not aromatics or cycloparaffins.

4.1.4 *Yarrowia lipolytica* degradation

This organism, unlike the others described thus far, is a yeast. It is also a normal alkane degrader, as shown in Figure 7, and this yeast degrades the heavier normal alkanes progressively more than the lighter normal alkanes. It does not appear to have any effect on the isoparaffins in general nor the aromatic compounds in the fuel.

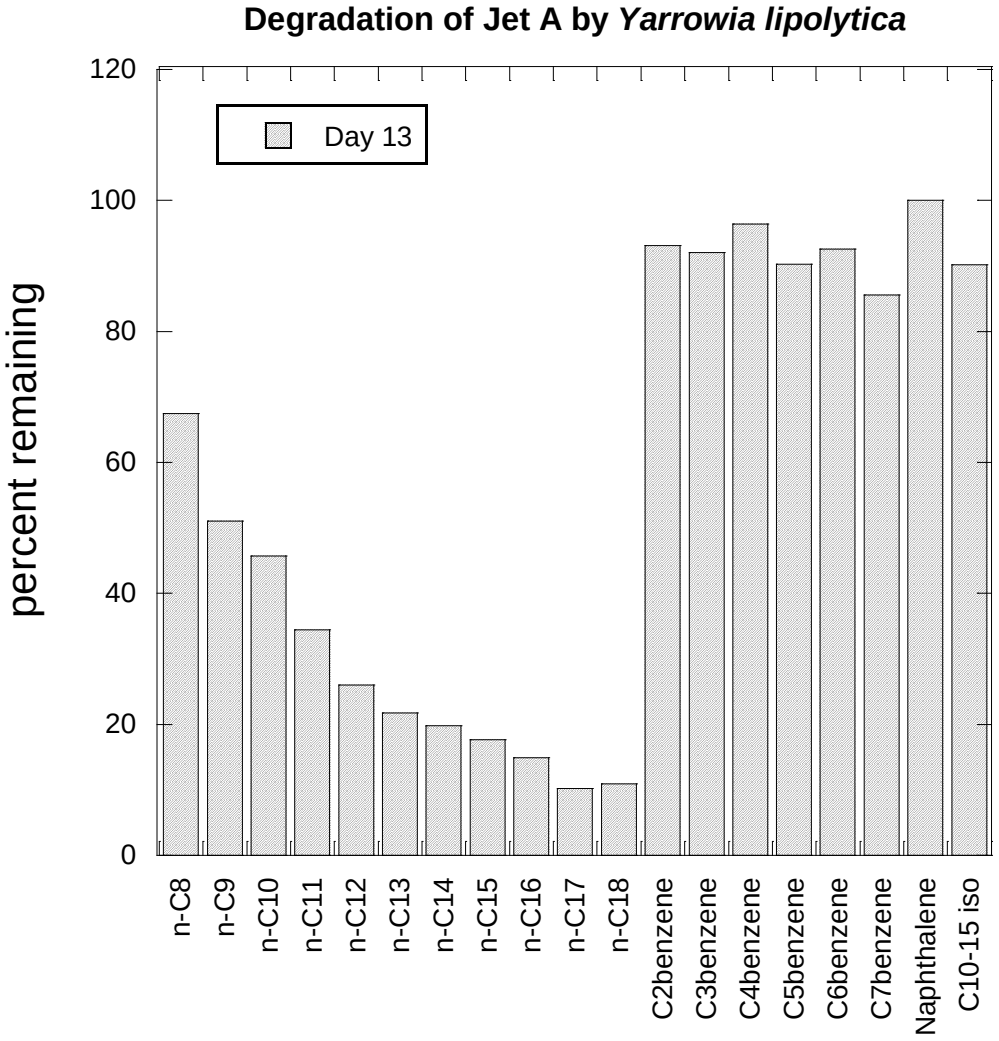


Figure 7. Degradation of Jet A by *Yarrowia lipolytica* in Jet A.

4.2 Summary of Vial Testing Results

A wide variety of other microbes were investigated using vial assays under this and other programs (Table 3). However, these 4 organisms shown above were chosen for larger scale testing because they represent more common microbial contaminants of fuel and fuel handling systems. Table 6 gives a summary of the compounds most preferred by each of the bacteria investigated. While the purpose of this program was to investigate the reaction of the specification test properties upon exposure to bacteria, this understanding of what compounds the bacteria consumed in each fuel was helpful to predict the properties that might be affected.

Table 6. Synopsis of microbial degradation assays and chemical degradation preferences of the microbes tested.

organism	changes observed in the fuel from microbial degradation
Pseudomonas aeruginosa	**Primary: C11 and higher n alkane degradation**
Marinobacter Hydrocarbono-clasticus	**Primary: C8-C11 n-alkane degradation;** **Secondary: toluene, ethylbenzene, certain C3-alkylbenzenes**
Acinetobacter venetianus	**Primary: C8-C23 n-alkane degrader;** **Secondary: methyl-substituted alkanes**
Yarrowia lipolytica	**Primary: C8-C23 n-alkane degrader**

4.3 Specification Test Results for larger scale experiments

In the previous section, we evaluated the chemical changes expected from exposing common biological contaminants to Jet A and F-76 fuels. Generally, small-scale vial testing indicated that microbes *do consume* the fuel to some extent. However, each bacteria or organism targets certain components of the fuel and not others. If enough degradation is allowed to occur, there would have to be a change in fuel properties, because compounds are being selectively removed from the mixture. For example, if chemical components of high volatility were consumed, flash point may be affected. If enough low volatility components are consumed without consuming high volatility components, the flash point would have to decrease. Similarly, it is important to understand that if microbes are specific to chemical compounds, additives present in the fuel at parts per million levels may be removed from the fuel. If aromatic compounds are removed from the fuel, eventually, aromatic levels may decrease. If this happens, would materials interaction properties such as o-ring swell, elastomer-fuel interactions be affected?

This project was conducted by first investigating the chemical changes in the fuel caused by microbial degradation using four common microbes, mostly through the vial assays as described above. Then, as we gained more understanding, larger test system were investigated - primarily, 1 liter Teflon bottles, to subject more fuel to microbial degradation. The amount of degradation obtained was monitored by the changes in chemical composition, primarily using GCxGC. The GCxGC was able to measure n-alkanes reliably, which was used as a gauge to determine the amount of degradation which occurred. GCxGC also uses a small amount of fuel (less than 50 µL can be used to conduct a measurement). The remaining fuel was typically

separated from the aqueous media and used in conducting specification tests or research tests similar to specification tests. Table 2 can be used to guide the reader through the experiments that were conducted. Each experiment conducted is coded as to a "DLA-XX" number, so that the results could be tracked. As the experiments continued, investigations of interactions of bacteria were performed. In these experiments, bacteria were added sequentially, or were used in consortia, and the purpose of the experiment was to determine how the growth of each could be affected by the other.

We did not automatically submit fuels to full specification tests, because it is difficult to make enough volume of fuel exposed to bacteria to submit. We therefore made smaller amounts of collectable fuel and then tested selected properties which we felt were important to address. Early work resulted in investigating the low temperature properties of fuel (freeze point, viscosity, etc.,) because of the significant n-alkane degradation. As we became more comfortable with exposing larger amounts of fuel, we tried to address more of the specification tests. In many cases, non-standard research methods were used in order to minimize the amount of fuel used to get a result, but these were always tested against control samples, exposed the same way as the test samples, but without bacteria. In this way, we investigated specification properties that might be affected in exposed fuel.

4.3.1 *Pseudomonas aeruginosa* in F-76 (DLA-01)

This experiment was conducted over a 40 day time period, using replicate samples of a 1 liter Teflon bottle containing 10 mL of F-76 fuel and 100 mL of M-9 media with *Pseudomonas aeruginosa*. The ratio of fuel to media was critical. In this case, the 1:10 fuel:media ratio is ten times more than the 1:100 ratio used in the vial testing. Obviously, more bacteria available directly affect the amount of fuel consumed. And as explained earlier, the less fuel available increases the chance that changes in the fuel can be observed. The 1:100 vial experiments were conducted for 14 days, while this 1:10 experiment was conducted for 40 days. There is a trade-off in several important variables for these experiments: microbial concentration (fuel:media ratio), the absolute amount of fuel used so that analytical methods may detect a change, and time of exposure. Growth curves were conducted using the 1:10 fuel:media ratio and compared to 1:100 ratios at the endpoint of a 40 day exposure, as shown in **Figure 8**. Even at a 1:10 ratio, there was significant change in the media OD measurement. Good growth for a long term experiment would be considered an OD reading of at least 1.0.

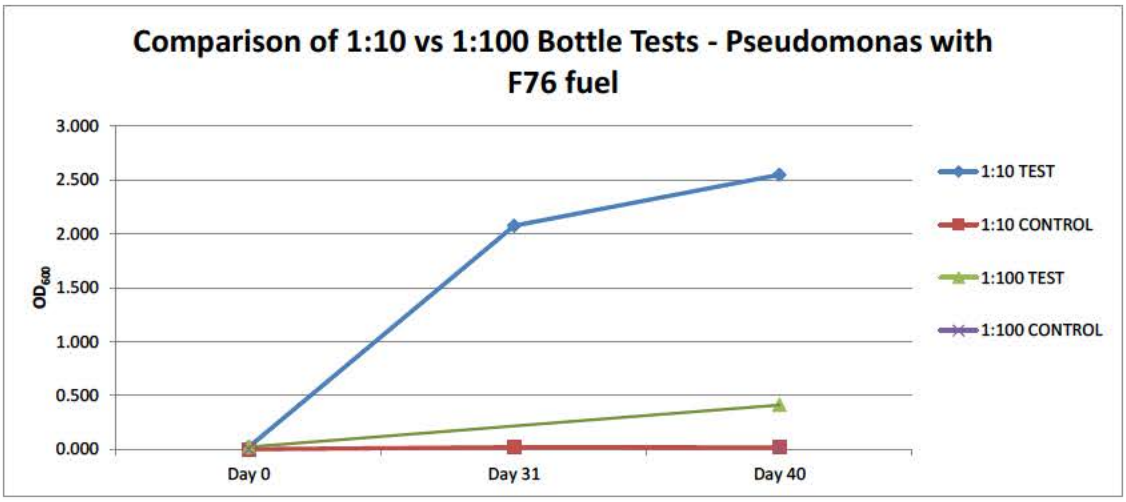


Figure 8. Growth curves for *Pseudomonas* in F-76 experiment in bottles (DLA-01). The 1:10 test produced more growth than the 1:100 experiment in bottles.

In order to evaluate the degradation of the fuel, and to be sure that the microbes were consuming the fuel in the same manner as in the vial experiments, fuel samples were taken from the bottles and analyzed using GCxGC. Tabular results for the DLA-01 experiment were plotted and graphical results are shown below (Figure 9). There was good agreement between the compounds consumed in the large scale experiment and previous vial experiments. The microbes show a preference for the heavier normal alkanes, as was the case in the vial experiments. However, since the large scale experiment contained more fuel and a smaller (1:10) ratio of fuel:media, the changes in fuel composition were less pronounced than in the vial experiments. However, compounds like n-hexadecane, present in the fuel at ~1.3 weight %, may be reduced in concentration by the bacteria by as much as 50% (down to 0.7 weight %) in this experiment as shown in Figure 9.

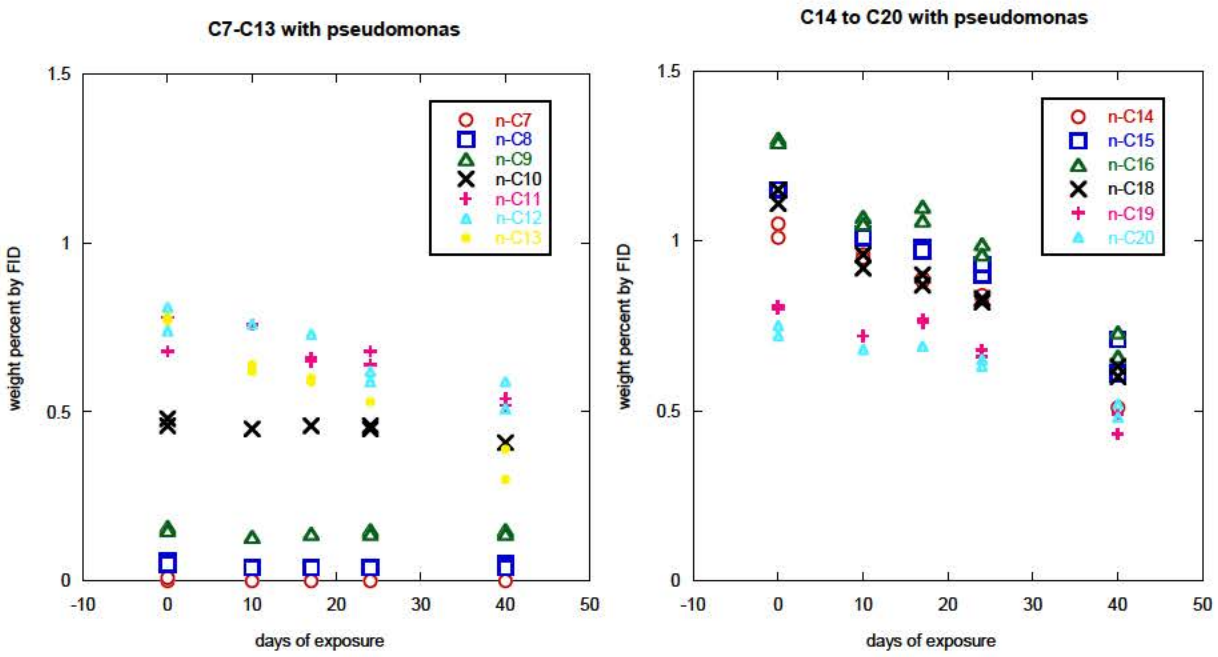


Figure 9. Normal alkanes (C7 – C20) in F76, exposed to *Pseudomonas aeruginosa* showing the preference of the microbes to consume the C14-C18 alkanes (DLA-01).

Over the 40 day period, the higher n-alkanes were clearly consumed preferentially to the lower normal alkanes, which should have affected freeze point (Striebich, et al., 2005). While F-76 does not have a freeze point specification, it does have a pour point and cloud point specification. **Table 7** shows the results for this experiment in terms of some of the specification tests chosen. First, it was clear that the control samples, which contained no bacteria, but were subjected to contact with the media, showed increased water content, but no increase in freeze point, cloud point or pour point. However, in the exposed replicate samples A and B, there is a clear change in low temperature properties as compared to the control. The normal alkane consumption, as expected, resulted in changes in low temperature properties related to freeze point. Freeze point improved by more than 6 °C (became lower, or more negative) due to the loss of n-alkanes; pour point and cloud point were also lowered. Density and water content were not

affected significantly by the bacteria; water content in the fuel is affected by the fact that fuel was exposed to a water layer and therefore is saturated with water in both cases.

We hypothesized that properties like Total Acid Number (TAN) would increase due to biological growth, but they did not increase significantly, even though the growth showed an OD reading of more than 2.5. The specification level for TAN in F-76 is 0.30 mg KOH/g, so the levels detected here are well below any specification test failures. It is possible that acids that may have formed from biological growth transferred to the media phase during the experiment. Organic acids are weak acids and have the ability to change the TAN of a fuel, if they are soluble in the fuel phase. The TAN measurement, which is a titration of the fuel with a potassium hydroxide solution, would respond if there were significant organic acids in the fuel. It did not respond significantly in this experiment, despite the high level of growth.

Table 7. Specification results for DLA-01: low temperature properties, density and water content for F76.

	FP (°C)	Cloud Point, °C	Pour Point, °C	density (g/mL)	water content, ppm	total acid number, mg KOH/g
F76 Neat	-11.7	-14.1	-18	0.851	not measured	0.068
Exposed A	-19.1	-20.8	-27	0.852	53.4	0.070
Exposed B	-17.6	-18.6	-24			
Control A	-11.9	-14.3	-18	0.857	51.3	0.065
Control B	-12	-14.3	-18			

Table 8 shows that the aromatic levels for this F-76 fuel changed somewhat. There was an actual increase in aromatic level in the value as the fuel was exposed to the bacteria. This ~2% (absolute) change in the total aromatics is likely a function of the decrease in the normal alkanes. By lowering the n-alkane through microbial degradation, the aromatic content as measured by ASTM D6379 is artificially elevated. If the aromatic level were close to specification limits, the

increase in aromatics due to alkane reduction could cause specification test failure for this property. Only an unrealistic amount of biodegradation would be enough reaction to cause aromatics to increase significantly; no significant aromatic product formation is occurring in these fuels. Only the increase in aromatics associated with the decrease in n-alkanes is occurring.

Table 8. Results of DLA-01 for aromatic content as measured by ASTM D6379.

	F76	**F76** Control A 5/28/2013	**F76** Control B 5/28/2013	**F76** Culture A 5/28/2013	**F76** Culture B 5/28/2013
D6379 (volume%)					
Monoaromatics	19.1	19.1	19.7	20.9	20.3
Diaromatics	3.3	3.4	3.4	3.6	3.6
Triaromatics	0.6	0.6	0.6	0.7	0.6
Total Aromatics	23.0	23.1	23.7	25.2	24.5

4.3.2 *Pseudomonas aeruginosa* in F-76 and Jet A (DLA-02)

This experiment was similar to DLA-01, except that two different fuels were used in separate experiments. Also, multiple bottles using a 1:10 fuel:media ratio for each fuel were used so that more fuel could be collected to conduct additional specification testing. There were ten 1 Liter bottles for each condition (exposed and control); the multiple bottles were collected at the conclusion of the test in a separatory funnel and the media layer drained off to allow the collection of a larger volume of fuel (typically, 60-80 mL). Other experiments conducted in this time period provided insight about the importance of oxygen concentration in the bottles as the bacteria aerobically degraded the fuel. These bottles were all uncapped at fixed intervals (every 7 days) during the 28 day experiment, in order to insure high growth rates for the *Pseudomonas*.

In addition, the media was often changed during longer experiments to give microbes a chance to grow as much as possible. **Figure 10** shows the growth curves for the two different fuels. **Table 9** gives the results for aromatic content as measured by ASTM D6379; **Table 10** gives other specification property results including acid number, viscosity, sulfur content and simulated distillation. These results show that the properties investigated did not change significantly with the exposure to microbes. Even though some low temperature properties like freeze point may have been improved by the disappearance of normal alkanes, other low temperature properties such as viscosity did not change at all. Similarly, there were no significant changes in the distillation properties as measured by simulated distillation. Even though the microbes may have preferred heavier normal alkanes, the small amount of change that this preference created did not cause a shift in distillation range which would show up in the specification test. Sulfur compounds were also unaffected, as the bacteria preferred to eat the alkanes. There are small differences shown in amounts of sulfur compounds detected which reflect the accuracy of the technique. In these results, there was no significant change in sulfur response due to any of the microbes.

Figure 11 and 12 shows thermal stability results for F76 and Jet A, respectively, as measured by the Quartz Crystal Microbalance (QCM). The QCM is a research technique for measuring thermal oxidative deposition with a highly sensitive quartz crystal. The oscillation changes that occur as the experiment proceeds is monitored and transferred into a mass accumulation on the crystal. Both the Jet A and the F-76 showed decreases in deposition level from the neat sample to the control sample. This change may have occurred as oxygenates or polar nitrogen compounds may have been extracted from the fuel as the fuel was exposed to media, even without bacteria. Then, comparing microbial exposure to the control, the deposition

rate due to the bacterial exposure increased for the F76 and was unchanged with the Jet A fuel. F-76 is inherently less stable than Jet A.

The F-76 control sample showed deposition of approximately 15 μg/cm^2 after 20 hours (**Figure 11**). The F-76 exposed to microbial degradation showed 25 μg/cm^2 for the same amount of time. This could indicate an effect due to the microbes, but the deposition levels are so high that it is difficult to say with certainty that the effect is microbial. The Jet A results (**Figure 12**) showed essentially no change due to subjecting fuel to biological degradation.

As was suspected initially, the specification tests which emphasize trace properties, like thermal stability or existent gum, might be susceptible to the bacterial contamination as compared to the bulk properties (simulated distillation, percent sulfur, percent aromatics, etc.). Thermal stability tests such as JFTOT may also be susceptible to bacterial contamination because of the effect of trace properties on thermal stability.

Table 9. Aromatic content as measured by ASTM D6379, a low sample volume substitute for ASTM D1319 aromatics specification test (DLA-02).

	JetA 4658 Control 7/18/2013	JetA 4658 Exposed 7/18/2013	F-76 Control 7/18/2013	F-76 Exposed 7/18/2013
D6379 (volume%)				
Monoaromatics	16.8	17.5	18.6	20.6
Diaromatics	1.4	1.4	3.5	3.8
Triaromatics			0.6	0.7
Total Aromatics	18.2	18.9	22.7	25.1

In these experiments, we also examined polar content in the exposed fuel, using Solid Phase Extraction (SPE) to evaluate whether trace components in the fuel changed by being exposed to bacteria. In addition, the aqueous media part of the sample was also tested using Stir-Bar Sorptive Extraction (SBSE) to determine if any significant amount of organic compounds or fuel components could be observed in the water. Results for the media extraction did not show any observable or measureable components in the aqueous phase. The water was extracted using a miniature stir-bar (Twister-Gerstel) and magnetic stirrer at 800 rpm, to remove the organics into the polysiloxane phase on the outside of the magnetic stir bar. Then, the bar was extracted using methylene chloride to remove any extracted material. No significant components from the aqueous phase were observed that could be attributed to the exposure with *Pseudomonas*.

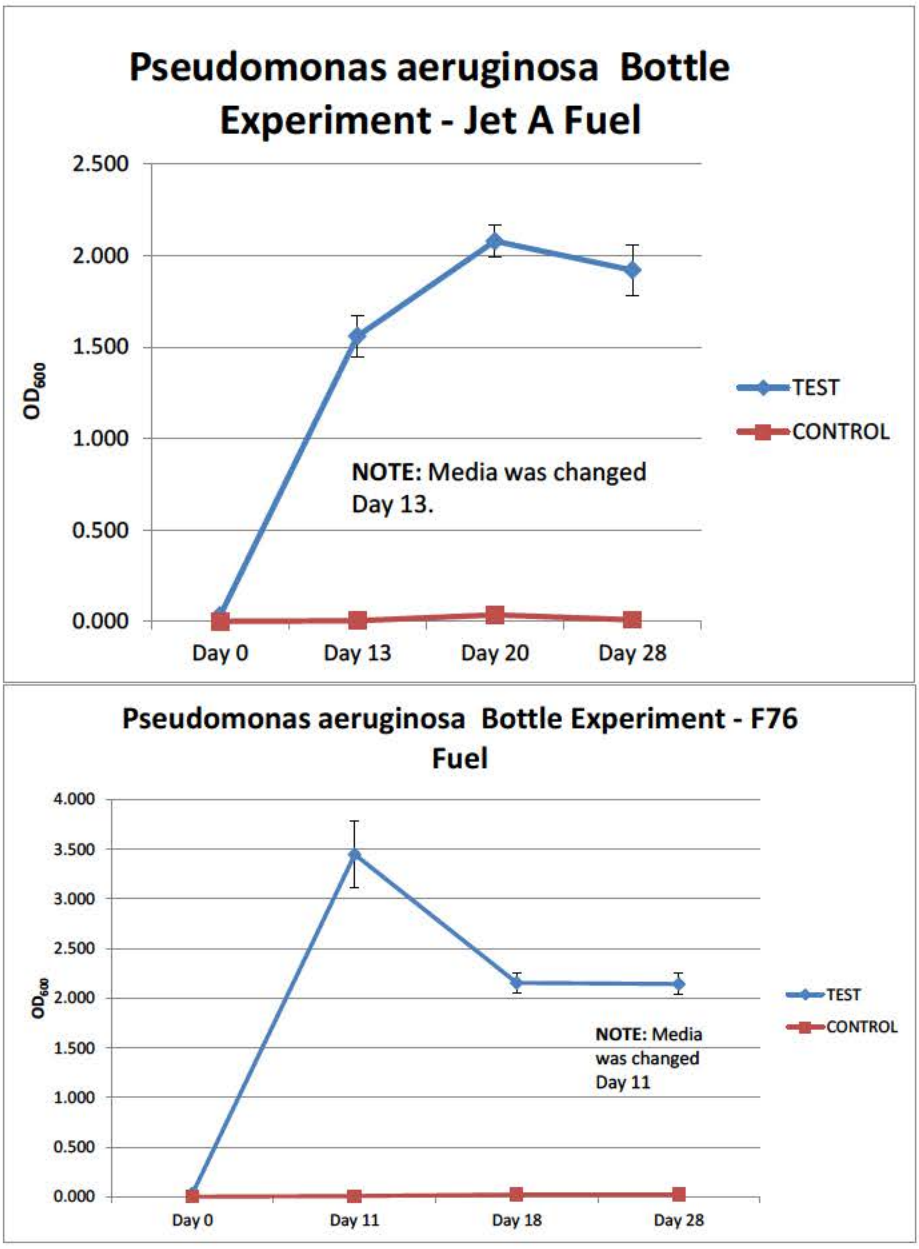


Figure 10. Growth curves for DLA-02, the exposure of Jet A (top) and F76 (bottom) to *Pseudomonas aeruginosa* in bottle testing.

Table 10. Specification testing conducted for Jet A and F76 exposed to *Pseudomonas* (DLA-02).

	JetA 4658 Control 7/18/2013	JetA 4658 Exposed 7/18/2013	F-76 Control 7/18/2013	F-76 Exposed 7/18/2013
Sulfur (GC-FPD), ppm (wt)	582	570	445	463
Viscosity, -20°C, cSt	5.17	5.16	-	-
Freeze Point, °C	-47.4	-51.6	-	-
Total Acid Number (mg KOH/g)	-	-	0.065	0.088
Simulated Distillation, D2887				
IBP, 0.5% recovered	115	115	142	142
5% recovered	150	150	186	185
10% recovered	166	166	203	204
15% recovered	174	174	216	218
20% recovered	183	182	231	230
25% recovered	190	189	241	240
30% recovered	196	196	251	250
35% recovered	201	200	260	258
40% recovered	208	207	268	268
45% recovered	214	212	276	275
50% recovered	216	216	284	282
55% recovered	223	222	291	290
60% recovered	229	226	299	297
65% recovered	235	234	304	304
70% recovered	238	238	312	311
75% recovered	245	245	318	318
80% recovered	253	252	328	327
85% recovered	259	259	338	337
90% recovered	269	268	350	350
95% recovered	301	281	367	367
FBP, 99.5% recovered	314	315	410	410

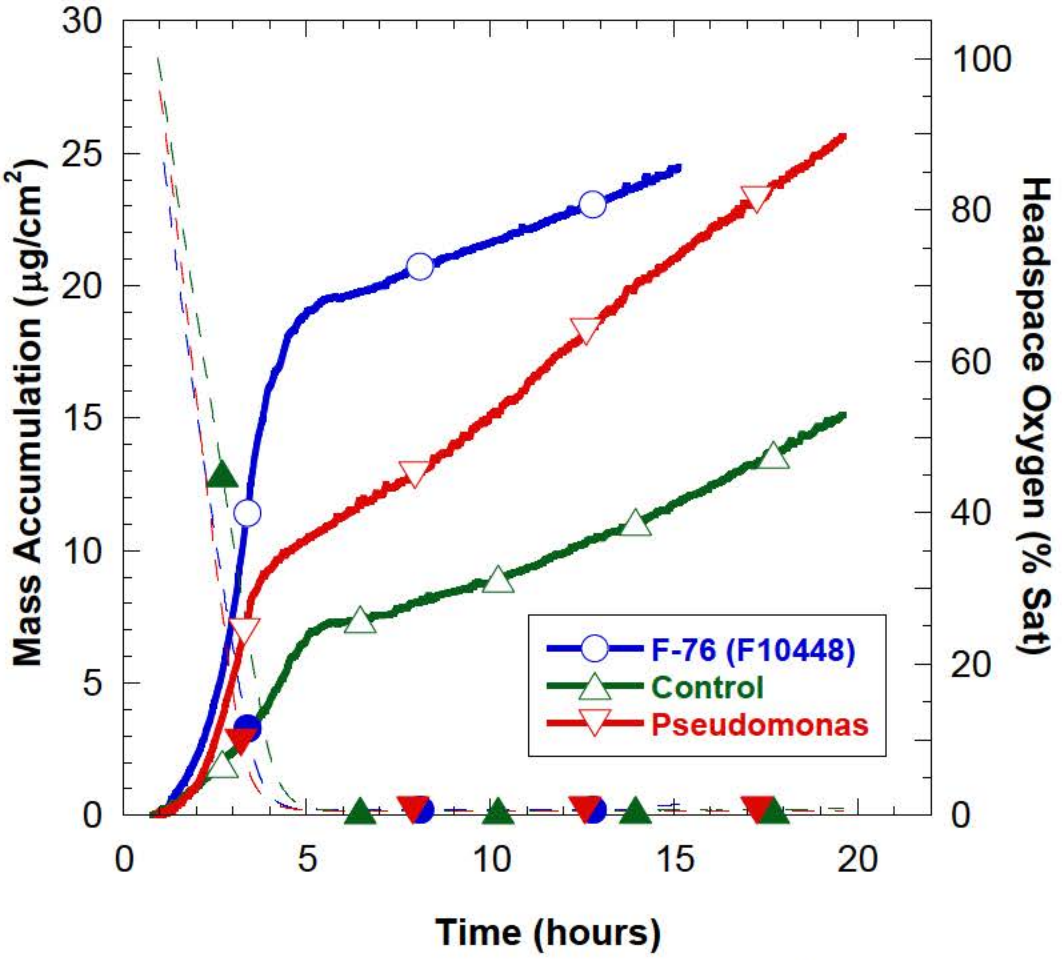


Figure 11. QCM profiles at 140°C of mass accumulation (solid lines – open markers) and headspace oxygen (dashed lines – closed markers) of F-76 diesel fuel (F10428) before and after 1 month exposure to *Pseudomonas* or a control.

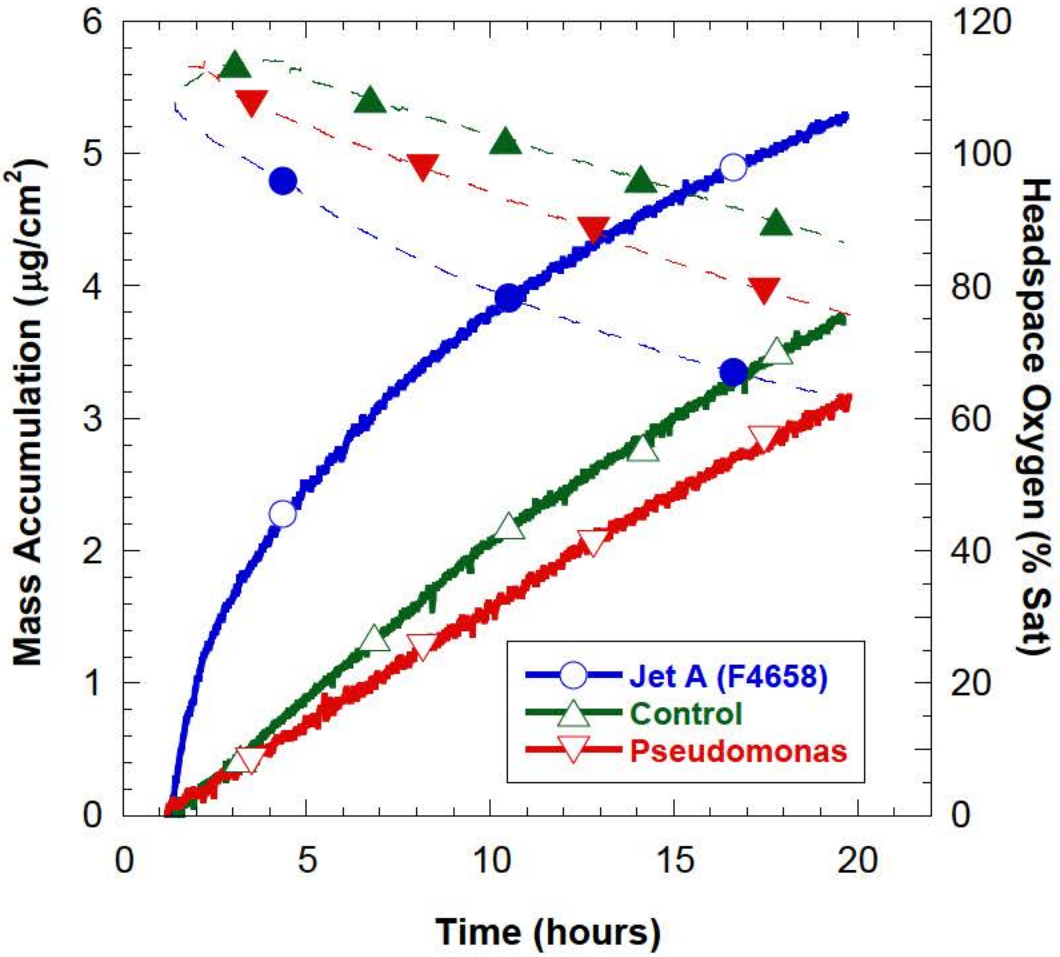


Figure 12. QCM profiles at 140°C of mass accumulation (solid lines – open markers) and headspace oxygen (dashed lines – closed markers) of Jet A (F4658) fuel, and the same fuel after 1 month exposure to *Pseudomonas* or a control.

The SPE extraction results were performed on the fuel material, with a 10:1.5 concentration of the fuel into a polar solvent – methanol. The results are shown in Figure 13 for both the Jet A and F76 fuels. Among the interesting polar components detected in the fuel which appeared to be introduced by the action of bacteria were substituted cyclohexenes. While a wide variety of these compounds appeared to be formed, we do not know the process by which they are produced given that there are no previous reports showing a mechanism for formation of these alkenes. Furthermore, at this moment we do not understand whether or not these compounds might contribute to thermal instability.

While SPE is not a specification test, it may be indicative of thermal instability, especially in the case of polar aromatic compounds like alkylphenols. Increasing the amount of phenols present in fuel is often related to less-thermally-stable fuel (Balster et al, 2006). While the amount of polars passed into jet fuel by bacteria is likely very small, even small amounts of polars can affect thermal and storage stability properties, as simulated using the QCM, especially if these polar components are aromatic (Balster et al, 2006).

GCxGC output for the analysis of fuel that has been stressed by exposing it to *Pseudomonas* bacteria for a period of 28 days is provided in **Table 11**. Both Jet A and F76 were analyzed to determine the changes in each fuel after the exposure. Jet A showed a 5% (absolute) change in the normal alkane concentration and F76 showed a greater than 8% change. This type of change will affect (and did in fact show) a change in the low temperature characteristics of the fuel. Generally, the loss of normal alkanes has improved the low temperature properties of the fuel.

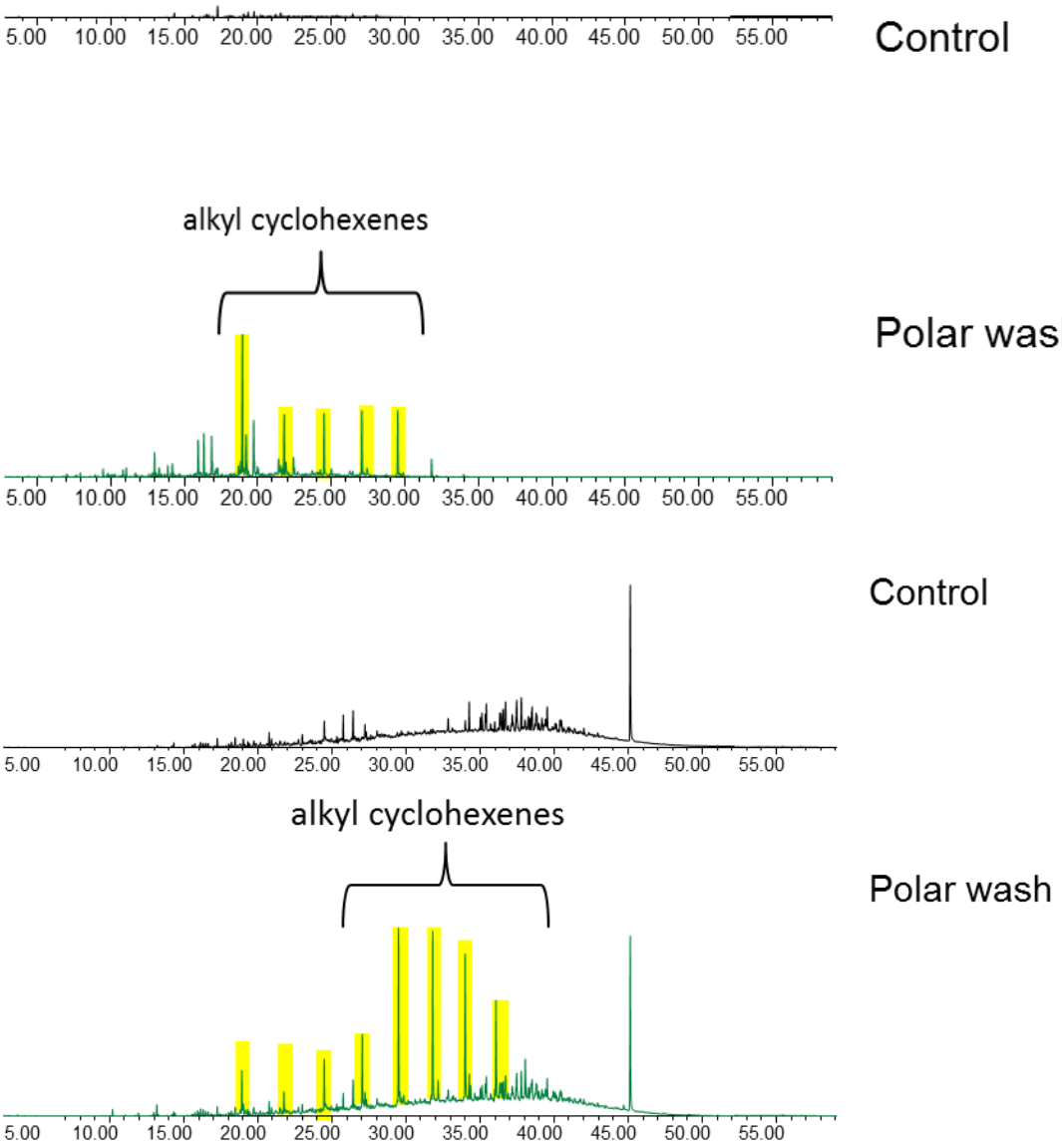


Figure 13. Top panel: solid phase extraction of the control sample and the *Pseudomonas* exposed to Jet A. Bottom panel: solid phase extraction of the control sample and *Pseudomonas* exposed to F76. Both samples indicate the formation of alkylcyclohexenes as possible trace decomposition products of the normal alkane degradation in the fuel.

Table 11. GCxGC results for Jet A and F76 exposed to *Pseudomonas* aeruginosa for experiment DLA-02

	DLA Jet A control	DLA Jet A exposed	DLA F76 control	DLA F76 exposed
	Weight %	Weight %	Weight %	Weight %
Aromatics				
Total Alkylbenzenes	**13.18**	**13.57**	**7.20**	**7.55**
Total Alkylnaphthalenes	**1.44**	**1.47**	**2.13**	**2.50**
Total Cycloaromatics	**4.20**	**4.55**	**6.27**	**7.19**
Total Aromatics	**18.82**	**19.59**	**15.89**	**17.53**
Paraffins				
Total iso-Paraffins	**32.40**	**33.61**	**26.53**	**27.89**
n-Paraffins				
n-C07	0.13	0.12	<0.01	<0.01
n-C08	0.55	0.50	0.02	0.03
n-C09	1.23	1.21	0.15	0.16
n-C10	2.89	2.81	0.46	0.48
n-C11	4.07	3.75	0.82	0.75
n-C12	4.27	3.29	0.95	0.62
n-C13	3.24	2.06	1.05	0.45
n-C14	2.43	1.85	1.33	0.74
n-C15	1.39	1.07	1.72	0.93
n-C16	0.64	0.46	2.00	0.63
n-C17	0.24	0.16	2.35	0.79
n-C18	0.05	0.04	1.79	0.64
n-C19	0.02	0.01	1.30	0.67
n-C20	<0.01	<0.01	1.14	0.72
n-C21	<0.01	<0.01	0.82	0.51
n-C22	<0.01	<0.01	0.52	0.33
n-C23	<0.01	<0.01	0.30	0.18
n-C24	<0.01	<0.01	0.15	0.10
n-C25	<0.01	<0.01	0.07	0.04
n-C26	<0.01	<0.01	0.02	0.02
n-C27	<0.01	<0.01	<0.01	<0.01
Total n-Paraffins	**21.15**	**17.35**	**16.98**	**8.79**
Cycloparaffins				
Total Monocycloparaffins	**21.85**	**23.45**	**28.72**	**32.21**
Total Dicycloparaffins	**5.67**	**5.89**	**11.85**	**13.56**
Total Tricycloparaffins	**0.11**	**0.10**	**0.03**	**0.02**
Total Cycloparaffins	**27.63**	**29.45**	**40.60**	**45.79**

4.3.3 *Pseudomonas aeruginosa* in F-76 (DLA-03) exposed in a Bio-bag

In order to expose large samples of fuel to bacteria under the same 1:10 fuel:media ratios as we have been performing on other smaller systems, a 50 Liter polypropylene bio-bag was used to contain the media and fuel. This system produced approximately 1 liter of fuel which could then be submitted for specification testing for many of the F76 properties. The bag contained 25 liters of air, 10 liters of media and 1 liter of fuel when the experiment began. The difficulty in performing this experiment is largely in the sample container. In this case, we obtained a "bio-bag" (ThermoFisher) a plastic bag with three ports for adding and removing fuel, water and air (**Figure 14**).

Figure 14. Bio-bag container (foreground) provided large surface area for conducting long term experiments with biological media and fuel.

Because the microbe being tested was *Pseudomonas*, there was a constant concern about the oxygen concentration for the system. After 2 weeks, the headspace of the bag was measured to be 10% oxygen and the media OD was 0.35 at 600 nm. Fresh air was replaced in this experiment by pulling out 10 liters of headspace with a 1 liter gas syringe and replacing with fresh air. Media was replaced after 6 weeks to insure that the growth was as fast as possible, even though the OD readings did not increase significantly above the 0.35 level. A summarized version of the GCxGC testing is shown in **Table 12** below.

Overall, the experiment was not a great success. A successful experiment in this project is one in which the OD readings are high (greater than 1.0 OD), indicating fast growth for the microbe. Because this experiment was conducted in a sealed container, i.e., the sealed biobag, the growth may have been limited because oxygen was not as available as it might otherwise be. While bottle experiments for *Pseudomonas* showed n-alkane concentrations being reduced between 3 and 5% absolute, these normal alkanes were only decreased less than 1 %. Since the food source was not limited, and the media was changed once during the experiment, we assumed that the reason for the slow growth was due to a lack of available oxygen. It was a dilemma for the experimenters, because ideally, the volatiles concentration will be affected if oxygen is bubbled or if the system is opened to atmospheric air for such a long period of time. Interestingly, the fact that available oxygen can be used up in biological growth in fuel tank systems, may be one of the best deterrents for fuel biodeterrioration although anaerobic processes such as biocorrosion may then take place. If bacteria consume the oxygen in a system, they will stop to actively grow and degrade the fuel, unless mass transport rates of oxygen from the fuel to the water phase are sufficient to resupply oxygen to the bacteria that is living at the fuel-water interface.

Table 12. Summarized results from the GCxGC experiments conducted for the "Bio-bag" exposure.

	Original F76	Exposed	Final Exposed	Foam fuel, a	Foam fuel, b
	7/3/2013	8/16/2013	9/11/2013	9/11/2013	9/11/2013
	Weight %	Weight %	Weight %	Weight %	Weight %
Aromatics					
Total Alkylbenzenes	11.68	11.88	11.99	11.94	11.89
Total Alkylnaphthalenes	7.15	7.13	7.25	7.20	7.16
Total Cycloaromatics	7.42	7.39	7.83	7.95	7.77
Total Triaromatics	0.88	0.89	0.90	0.90	0.88
Total Aromatics	27.14	27.29	27.98	27.99	27.71
Paraffins					
Total iso-Paraffins	25.65	26.03	25.37	25.43	25.60
n-C07	0.01	0.01	<0 01	<0.01	<0 01
n-C08	0.06	0.06	0.05	0 05	0.05
n-C09	0.21	0.21	0.20	0.19	0.20
n-C10	0.55	0.57	0.52	0 52	0.53
n-C11	0.79	0.80	0.80	0.79	0.81
n-C12	0.87	0.86	0.83	0 83	0.84
n-C13	0.84	0.81	0.75	0.75	0.75
n-C14	1.16	1.13	1.06	1 06	1.07
n-C15	1.47	1.42	1.37	1 36	1.37
n-C16	1.75	1.67	1.55	1 54	1.54
n-C17	1.98	1.77	2.33	2 33	2.33
n-C18	1.47	1.36	1.25	1 24	1.24
n-C19	1.17	1.13	1.02	1 02	1.02
n-C20	0.95	0.96	0.90	0 91	0.90
n-C21	0.68	0.63	0.62	0.64	0.62
n-C22	0.44	0.43	0.41	0.41	0.41
n-C23	0.27	0.25	0.23	0 24	0.23
n-C24	0.14	0.14	0.13	0.13	0.13
n-C25	0.07	0.06	0.06	0 06	0.06
n-C26	0.03	0.03	0.03	0 03	0.03
n-C27	0.01	0.01	0.01	0 01	0.01
Total n-Paraffins	14.93	14.30	14.12	14.10	14.13
Total Paraffins	40.59	40.34	39.50	39.53	39.73
Cycloparaffins					
Total Monocycloparaffins	23.48	23.84	24.10	24.09	24.19
Total Dicycloparaffins	8.75	8.49	8.38	8.35	8.33
Total Tricycloparaffins	0.05	0.04	0.05	0.04	0.04
Total Cycloparaffins	32.28	32.37	32.52	32.48	32.56

The purpose of this experiment was to expose the fuel to bacterial growth and to then collect the fuel and submit it for specification testing. Even though the amount of fuel degradation was not high as measured by normal alkane concentration decreases (**Table 12**), there was a liter of fuel available which was submitted for testing. The results for as many specification tests as could be done with the available fuel are provided in **Table 13**.

The results show that there are slight changes from the fuel being exposed to the bacteria. In spite of the effort taken to contain volatiles by having a sealed bio-bag, the flash point of the fuel increased from 72.5 to 76.5°C, likely due to some evaporation of volatile compounds.. The pour point also decreased (became more negative) in the exposed sample, probably due to the decrease in the normal alkane concentration; interestingly, the cloud point did not increase (improve) as the pour point did. Other low temperature properties (viscosity) were not affected.

Even though the volatiles appeared to be evaporated somewhat as measured by the flash point, this increase in flash point did not affect the distillation temperatures greatly. Specifically, there was not a large increase in the 10% recovered value. Sulfur content did not increase significantly from the neat F76 sample, even though the F76 sample was technically above the specification limit at the start of the test. None of the other properties changed at all as compared to the neat F76 fuel sample.

There is some research that specifies that microbial contamination will affect the corrosion inhibitor/lubricity improver (LI/CI) concentration (Stamper, et al., 2012). In this case, the lubricity remained essentially the same, increasing from 0.35 to 0.36 mm (wear scar). This insignificant change indicated that CI/LI reduction apparently did not occur in this experiment or other molecules produced by. In fact, overall, there were no significant changes in specification testing from this exposure.

Table 13. Specification test changes for F76 exposed to *Pseudomonas aeruginosa* in the bio-bag experiment (DLA-03).

Method	Test	Min	Max	Neat F76	Exposed F76
ASTM D4176-04	Appearance			Pass	Pass
ASTM D93-13	Flash Point, (°C)	60		72.5	76.5
ASTM D5773-10	Cloud Point, (°C)		-1	-10	-10
ASTM D5949-10	Pour Point, (°C)		-6	-12	-18
ASTM D445-12	Viscosity at 40°C	1.7	4.3	3	3.1
ASTM D86-12	Distillation				
	10% Recovered , (°C)	report		216	218
	50% Recovered , (°C)	report		278	280
	90% Recovered , (°C)		357	329	331
	End Point , (°C)		385	354	355
	Residue, (°C)		3	1.3	1.2
ASTM D130-12	Copper Strip Corrosion (2 h at 100 C)		1 (max)	1a	1a
ASTM D4294-10	Total Sulfur (% mass)		0.1	0.33	0.34
ASTM 524-10	Carbon Residue, 10% Bottoms (% mass)		0.2	0.05	0.04
ASTM D482-13	Ash (mass %)		0.005	0	0
ASTM D976-06	Cetane Index, Calculated	43		51	51
ASTM D974-12	Acid Number (mg KOH/g)		0.3	0.11	0.11
ASTM D7171-05	Hydrogen content by NMR, (% mass)			13.2	13.3
ASTM D4052-11	Density @ 15 C (kg/L)			0.845	0.845
ASTM D6045-12	Color, Saybolt			-16	-16
ASTM D7688-11	HFRR Lubricity @60C, Wear Scar diam (mm)			0.351	0.36

4.3.4 *Pseudomonas aeruginosa* in a carboy with Jet A (DLA-04)

A carboy is simply a plastic laboratory container, large in volume, used to hold liquids (**Figure 15**). This 2 gallon carboy was used to conduct a 90 day experiment, with the media changed on day 69. The OD readings for this experiment were slightly higher than the biobag experiment (DLA-03), as shown in Figure 16. Overall, the growth was reasonable (as compared to the biobag) in this experiment, with levels increasing to 0.70 OD over the course of the experiment (**Figure 16**). The exposure of the fuel to microbes appeared to extract some of the yellow color from the Jet A sample, which may indicate that polar compounds are being extracted from the original fuel (Figure 15- right panel).

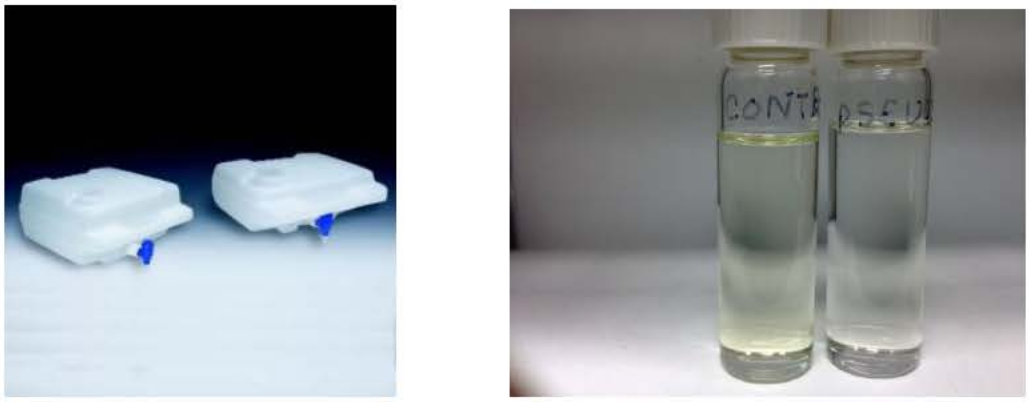


Figure 15. (Left) Color difference between samples exposed to *Pseudomonas* in carboy. There is a slight color change from the control sample (yellow) to the *Pseudomonas*-exposed sample (clear) (right). Carboys similar to the ones used in these experiments.

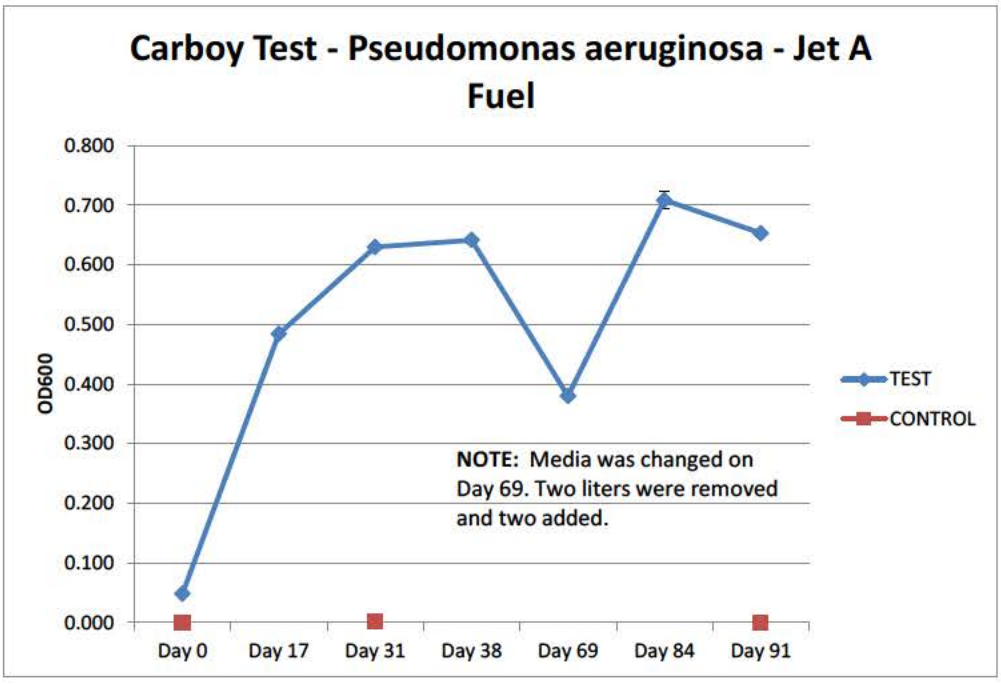


Figure 16. Carboy experiment growth curve for Jet A exposed to *Pseudomonas aeruginosa* (DLA-04).

The results from chemical testing of the fuel samples after the 90 day carboy test are shown in **Table 14**. In summary, the results show that approximately 2% of the n-alkanes were degraded by the *Pseudomonas*. In this experiment, 300 mL of fuel was used with 3 liters of M-9 media. The result of the 90 day carboy test was the generation of a 150 mL sample of Jet A fuel, with which several tests were conducted. **Table 15** shows the results of this experiment which indicated that the low temperature properties of the fuel were only affected in a positive way, in that the freeze point, pour point, cloud point and viscosity all become lower as compared to the control. The reason for this improvement is, again, that the normal alkanes are being consumed and normal alkane concentration (especially longer n-alkanes) is directly related to freeze point.

There was enough fuel produced in this exposure to allow the lubricity test to be conducted. The Ball-on-Cylinder Lubricity Evaluation (BOCLE) was conducted on both the neat Jet A fuel and the fuel exposed to 90 days of biodegradation in the carboy. The BOCLE test revealed a change in the wear scar diameter such that the degraded sample was lower in wear scar: the wear scar improved from 0.57 mm for the original sample to 0.41 mm for the degraded sample. This result indicates an improvement in lubricity after exposure to the microbes. This result was surprising because literature studies concerning the biodegradation of corrosion inhibitor/lubricity improver (CI/LI) additive have been conducted (Stamper et al., 2012). This result did not measure CI/LI concentration, but the property most affected by CI/LI concentration, i.e., lubricity. Perhaps the biofilm produced in this exposure had surfactant and lubricity properties and served to decrease wear scar, therefore improving fuel lubricity.

Table 14. Summarized results from the GCxGC experiments conducted for the "Carboy" exposure.

	carboy control 4658	carboy exposed 4658
	Weight %	Weight %
Aromatics		
Total Alkylbenzenes	**14.30**	**14.62**
Total Alkylnaphthalenes	**0.85**	**0.86**
Total Cycloaromatics	**4.63**	**4.74**
Total Aromatics	**19.78**	**20.22**
Paraffins		
Total iso-Paraffins	**34.18**	**34.71**
n-Paraffins		
n-C07	0.02	0.02
n-C08	0.17	0.17
n-C09	0.84	0.85
n-C10	3.29	3.33
n-C11	6.20	6.07
n-C12	5.48	4.95
n-C13	3.37	2.72
n-C14	1.80	1.57
n-C15	0.76	0.66
n-C16	0.31	0.26
n-C17	0.15	0.11
n-C18	0.03	0.03
n-C19	<0.01	<0.01
n-C20	<0.01	<0.01
n-C21	<0.01	<0.01
n-C22	<0.01	<0.01
n-C23	<0.01	<0.01
Total n-Paraffins	**22.44**	**20.78**
Cycloparaffins		
Total Monocycloparaffins	**19.52**	**20.01**
Total Dicycloparaffins	**4.01**	**4.22**
Total Tricycloparaffins	**0.06**	**0.07**
Total Cycloparaffins	**23.59**	**24.30**

Table 15. Low temperature properties from exposure of Jet A to *Pseudomonas* for 90 days in a carboy.

Low temperature properties	**Jet A control**	**Jet A, Exposed 91 days**
Freeze Point, °C	-50.3	-53.1
Cloud Point, °C	-50.4	-53.2
Pour Point, °C	-58.5	-57
Viscosity (-20 °C), cSt	5.00	4.95
Total Acid Number (TAN), mg KOH/g	<0.003	0.004
Lubricity, wear scar diameter, mm	0.57	0.41

Polar Compounds produced during Carboy Experiments.

In addition to the specification testing, we have tried whenever possible to perform non-specification test to determine what happens to the samples as they become exposed to bacteria. Solid phase extraction (SPE) was carried out for the fuel phase of the 300 mL that was exposed to the *Pseudomonas* microbe. We introduced 20 mL of sample through an SPE cartridge under conditions described in previous work, and eluted the polar compounds from the 20 mL of sample into 1.5 mL of methanol solvent. Consequently, the only compounds that should be observed from the mixture are the low concentration of compounds that are polar in the fuel. Earlier, we observed that compounds like the alkyl cyclohexenes were present as low concentration impurities in the products of the fuel after exposure to *Pseudomonas* in plastic Teflon bottles, from either F76 or Jet A. These same compounds were again observed from the experiment conducted in DLA-04 with the Carboy. However, other compounds were present in addition to alkyl cyclohexenes including a variety of alcohols and ketones which are common n-

alkane biodegradation products. The most abundant of these compounds were the 5- and 6-substituted long chain alcohols, which resulted from a normal alkane being processed by an organism to an alcohol. This finding is consistent with the proposed mechanism for decomposition of n-alkanes via oxygenase to primary and secondary alcohols. While primary alcohols are metabolized to the corresponding aldehyde and rapidly converted into a fatty acid for degradation, the sub-terminal oxidation of n-alkanes to secondary alcohols (Kotani et al. 2007) followed by conversion to their corresponding ketones which are finally oxidized by a Baeyer–Villiger monooxygenase to render an ester may be a slower process that allows for detection of the ketones intermediates using GC-MS. An example of a chromatogram showing these products is provided in **Figure 17**.

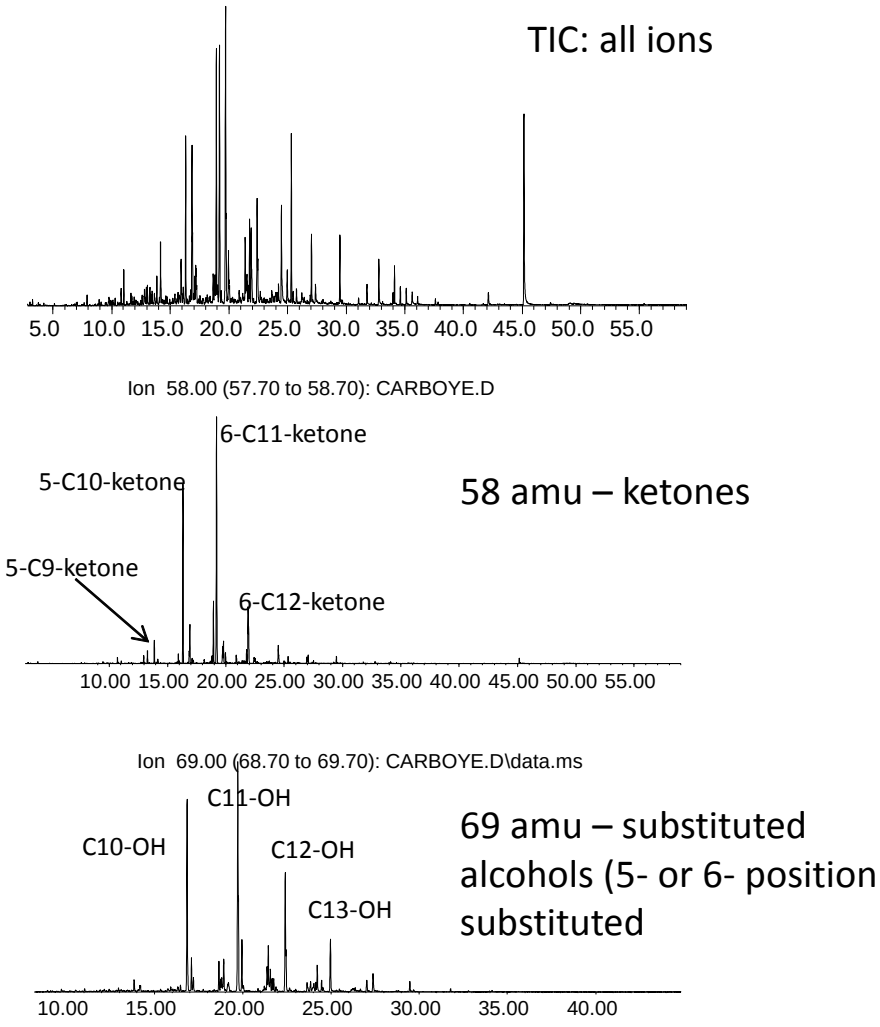


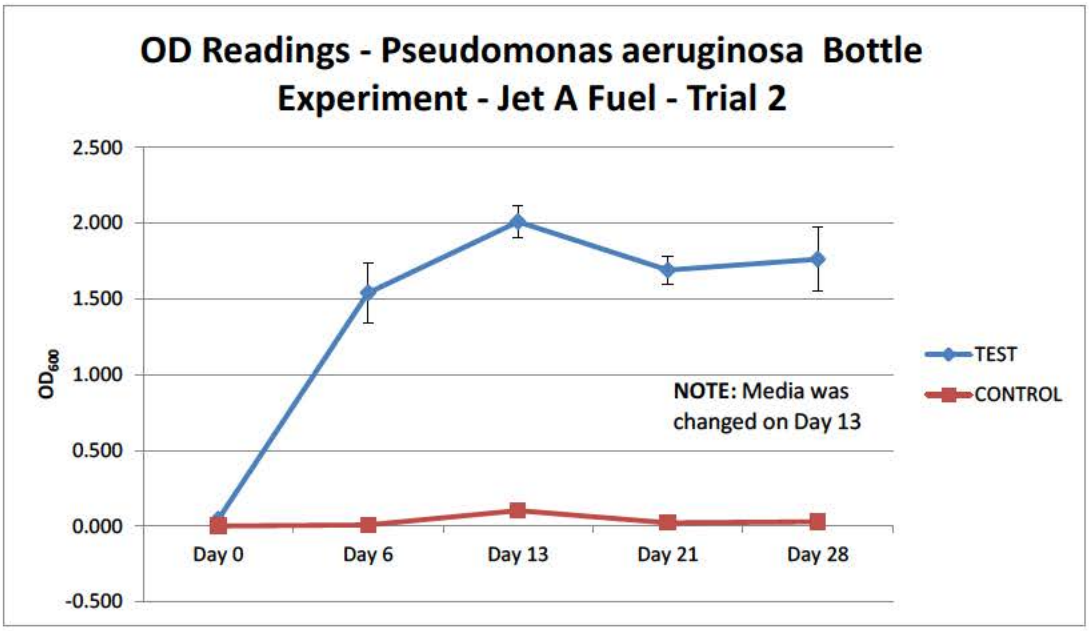
Figure 17. GC-MS chromatograms used to determine the products of reaction from bacterial degradation of normal alkanes in Jet A fuel from *Pseudomonas*. The compounds represent the polar part of the sample. The polars were obtained from the exposure of Jet A to *Pseudomonas* in a carboy.

4.3.5 *Pseudomonas aeruginosa* in a plastic Teflon bottles with Jet A and F-76 (DLA-05)

It was desired to replicate the experiments conducted in DLA-02, an important experiment to determine how fuel would react to realistic levels of bacteria. These experiments were conducted in bottles with weekly venting, to let oxygen back into the system and the media

changes half way through the experiment, at 14 days. Growth curves are shown in **Figure 18** for Jet A and F76. GC results are summarized in **Table 16 and 17** for Jet A and F76 respectively. They match well the results from DLA-02. They both show significant degradation of the normal alkanes as the bacteria degrade the fuel sample. The amount of fuel collected after exposure in the bottles was surprising for this sample. While 80-100 mL was expected, the actual amount collected was about 35 mL for the Jet A and about 53 mL for the F-76.

Corrosion Inhibitor/Lubricity Improver (CI/LI) is of significant interest in fuel because other authors have observed decreases in CI/LI concentrations due to bacterial consumption. Enough sample was available after this study (DLA_05) to conduct the analysis to evaluate fuel lubricity – BOCLE. The results for this test are provided in Table 18. These results indicate that the lubricity of the fuel is not significantly affected by the introduction of bacteria under the conditions of the experiment. In one case, Jet A exposed to bacteria was shown to improve in lubricity properties, probably due to the formation of trace amount of polar components from the bacteria. However, data exists from this effort that indicates that the bacteria can consume corrosion inhibitor. More work is needed to understand the relationship between lubricity and bacterial degradation. While corrosion inhibitor may be consumed during microbial degradation, degradation products may provide more lubricity. These forces act against each other, making a simple explanation of process very difficult. Future studies may address this topic.



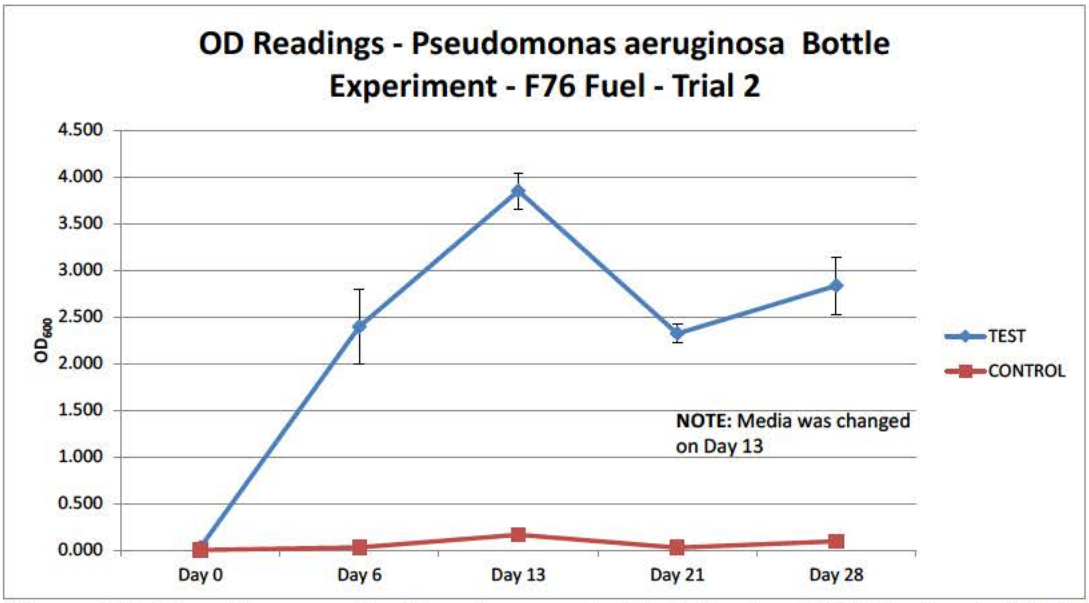


Figure 18. Growth curves for *Pseudomonas* in Jet A (top) and F76 (bottom). (DLA-05)

Table 16. Jet A exposure in *Pseudomonas* (DLA-05)

	POSF-4658 neat	Jet A (4658) DLA-05 control b	Jet A 4658 dla05 control a	Jet A 4658 exposed pseudo DLA-05
	Weight %	Weight %	Weight %	Weight %
Aromatics				
Total Alkylbenzenes	**13.69**	**13.53**	**13.54**	**14.11**
Total Alkylnaphthalenes	**1.76**	**1.81**	**1.83**	**1.89**
Total Cycloaromatics	**5.79**	**5.89**	**5.90**	**6.20**
Total Aromatics	**21.25**	**21.23**	**21.28**	**22.20**
Paraffins				
Total iso-Paraffins	**31.34**	**31.08**	**31.06**	**32.28**
n-Paraffins				
n-C07	0.15	0.10	0.10	0.10
n-C08	0.54	0.47	0.46	0.44
n-C09	1.14	1.08	1.06	1.07
n-C10	2.55	2.52	2.50	2.47
n-C11	3.62	3.73	3.72	3.40
n-C12	3.70	3.77	3.77	2.89
n-C13	2.86	2.94	2.92	1.72
n-C14	2.17	2.21	2.23	1.60
n-C15	1.28	1.29	1.30	0.96
n-C16	0.61	0.59	0.60	0.40
n-C17	0.27	0.27	0.27	0.16
n-C18	0.05	0.05	0.05	0.04
n-C19	0.02	0.02	0.02	0.01
n-C20	<0.01	<0.01	<0.01	<0.01
n-C21	<0.01	<0.01	<0.01	<0.01
n-C22	<0.01	<0.01	<0.01	<0.01
n-C23	<0.01	<0.01	<0.01	<0.01
Total n-Paraffins	**19.00**	**19.05**	**19.03**	**15.28**
Cycloparaffins				
Total Monocycloparaffins	**22.64**	**22.48**	**22.51**	**23.77**
Total Dicycloparaffins	**5.73**	**6.12**	**6.07**	**6.41**
Total Tricycloparaffins	**0.05**	**0.05**	**0.05**	**0.05**
Total Cycloparaffins	**28.42**	**28.64**	**28.63**	**30.23**

Table 17. F-76 exposure in *Pseudomonas* (DLA-05)

	F76 - 10304 (original)	DLA-05 F76 control	DLA-05 exposed
	7/3/2013	9/13/2013	9/13/2013
	Weight %	Weight %	Weight %
Aromatics			
Total Alkylbenzenes	**11.68**	**11.69**	**12.12**
Total Alkylnaphthalenes	**7.15**	**7.35**	**7.72**
Total Cycloaromatics	**7.42**	**7.63**	**7.96**
Total Triaromatics	**0.88**	**0.96**	**1.00**
Total Aromatics	**27.14**	**27.62**	**28.80**
Paraffins			
Total iso-Paraffins	**25.65**	**24.71**	**25.59**
n-Paraffins			
n-C07	0.01	<0 01	<0.01
n-C08	0.06	0.03	0.03
n-C09	0.21	0.13	0.14
n-C10	0.55	0.39	0.41
n-C11	0.79	0.64	0.61
n-C12	0.87	0.73	0.52
n-C13	0.84	0.76	0.45
n-C14	1.16	1.10	0.79
n-C15	1.47	1.49	1.00
n-C16	1.75	1.80	0.96
n-C17	1.98	2.71	1.82
n-C18	1.47	1.48	0.86
n-C19	1.17	1.34	0.83
n-C20	0.95	1.05	0.78
n-C21	0.68	0.74	0.52
n-C22	0.44	0.49	0.35
n-C23	0.27	0.29	0.19
n-C24	0.14	0.16	0.12
n-C25	0.07	0.07	0.05
n-C26	0.03	0.03	0.03
n-C27	0.01	<0 01	<0.01
Total n-Paraffins	**14.93**	**15.45**	**10.47**
Cycloparaffins			
Total Monocycloparaffins	**23.48**	**24.21**	**26.24**
Total Dicycloparaffins	**8.75**	**7.98**	**8.87**
Total Tricycloparaffins	**0.05**	**0.03**	**0.03**
Total Cycloparaffins	**32.28**	**32.22**	**35.14**

Table 18. Lubricity measurements for Jet A and F-76 exposed to *Pseudomonas* (DLA-05)

Fuel	Lubricity Wear Scar Diameter, mm	
Jet A	Neat	0.69
	Control (exposed to media)	0.57
	Exposed (Exposed to media and bacteria)	0.49
F-76	neat	ND
	Control (exposed to media)	0.514
	Exposed (Exposed to media and bacteria)	0.545

ND - not determined

4.3.6 *Marinobacter hydrocarbonoclasticus* in Hydroprocessed Renewable Diesel (HRD) fuel (DLA-06)

These experiments were conducted to repeat smaller vial experiments using a larger system. The marine bacteria *Marinobacter* showed slower growth than *Pseudomonas* in other fuels, and this fuel, HRD (hydroprocessed renewable diesel) was similar. In these experiments, there was only a small decrease in the lower n-alkanes (C8 through C12) where the other n-alkanes showed no differences at all. *Marinobacter* also degraded some aromatic compounds in conventional fuels, but because there were no aromatics in HRD, little degradation was observed. A plot of the growth curves for *Marinobacter* degradation of HRD is provided as **Figure 19**; data summarized from GCxGC experiments conducted for this fuel is given as **Table 19**.

The GCxGC data differs from the vial data obtained for this microbe. A chromatogram showing the volatile portion of a 1:100 fuel:media ratio vial experiment is shown in **Figure 20**. In this chromatogram, we observed some detail about what compounds were consumed by the Marinobacter which we were unable to determine in the 1:10 sample experiment conducted as DLA-06.

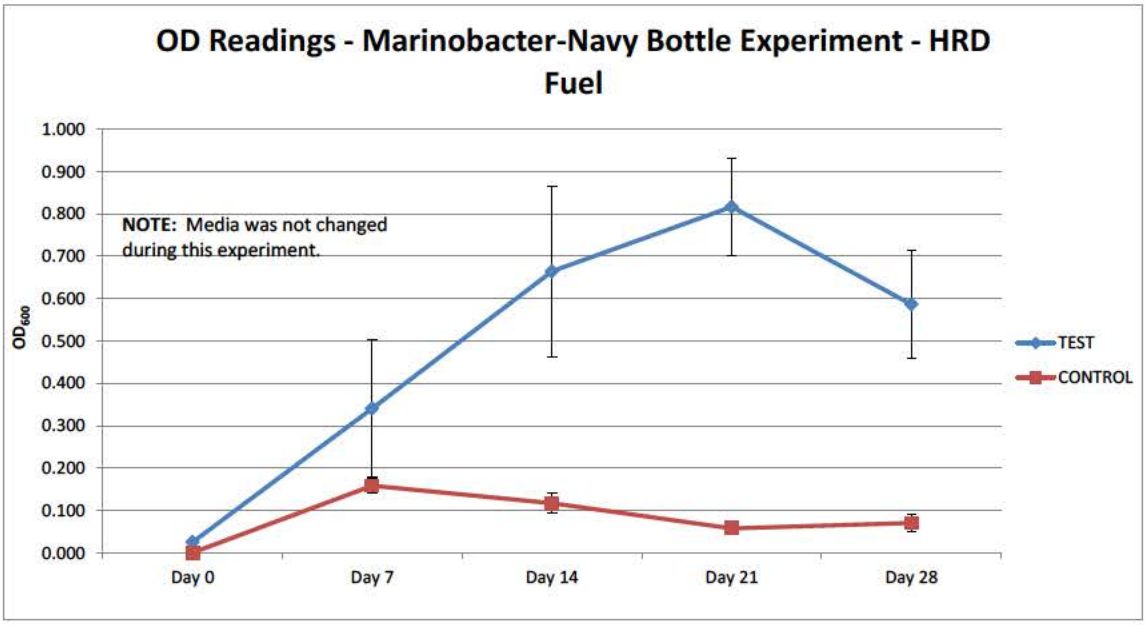


Figure 19. Marinobacter growth in HRD and M-9 media.

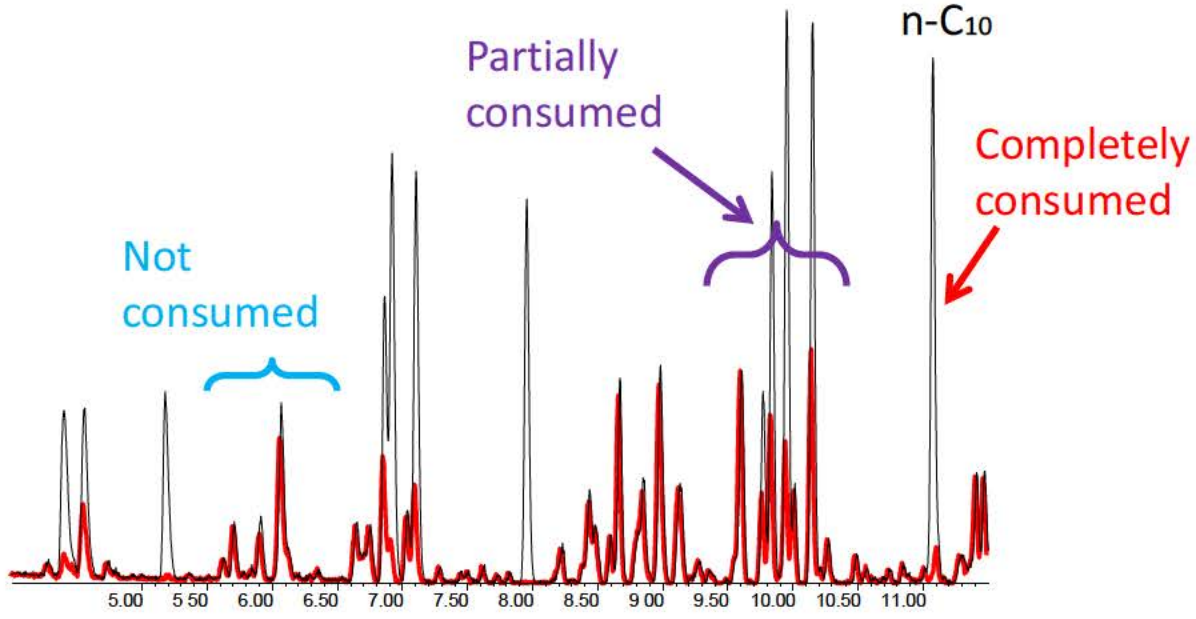


Figure 20. Two chromatograms of light components of HRD exposed to *Marinobacter* (red trace) and a control HRD sample (black) for 21 days. Figure shows examples of completely consumed n-alkanes, partially consumed mono-branched alkanes and did not consume multibranched alkanes. These results differ from bottle experiments due to the slow reaction rate for *Marinobacter*.

Table 19. HRD decomposition by *Marinobacter* in a 28 day bottle experiment. (DLA-06)

	DLA06 HRD Marinobacter control	DLA-06 HRD Marinobacter exposed
	Weight %	Weight %
Alkylbenzenes	0.1	0.09
Alkylnaphthalenes	0.09	0.09
Cycloaromatics	0.11	0.12
Total Aromatics	0.3	0.3
iso-Paraffins	82.65	82.83
n-Paraffins	16.69	16.50
total paraffins	99.34	99.33
Monocycloparaffins	0.22	0.24
Dicycloparaffins	0.15	0.14
Tricycloparaffins	<0.01	<0.01
Total Cycloparaffins	0.37	0.38

4.3.7 *Yarrowia lipolytica* with Jet A (DLA-07)

In screening experiments conducted prior to the larger scale bottle experiments, vials with *Yarrowia* in Jet A were examined for 7 days; no OD measurements were taken since this yeast tends to grow up the side of the bottles used. Even though the vials were not shaken in any way, the organisms seem to prefer a surface to grow on. However, they still consumed a good deal of the fuel, as shown in **Figure 21**, which is a comparison of a control sample (no yeast) after 7 days, and a *Yarrowia*-exposed sample after 7 days. Results dramatically show that, like many other organisms we have tested, *Yarrowia* shows a strong preference for the consumption of normal alkanes, and even after 7 days, consumes a large portion of the available alkanes. The

amount remaining for each normal alkane and corresponding error bars were quantified and are shown in **Figure 22**. On average, the normal alkanes are degraded by more than half. Interestingly, no branched alkanes nor aromatic compounds were degraded by this yeast.

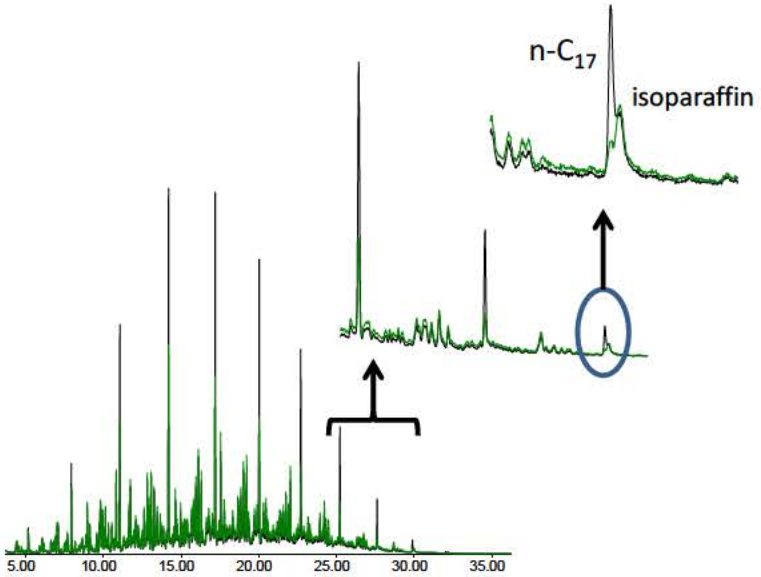


Figure 21. Screening studies showing the extraction of Jet A from the *Yarrowia*/media mix. The black tracing shows the Jet A control sample and the green tracing shows the 7 day exposure to *Yarrowia*. Note the difference between almost completely degraded normal C17, and the completely undegraded isoparaffins next to it, labeled "isoparaffins".

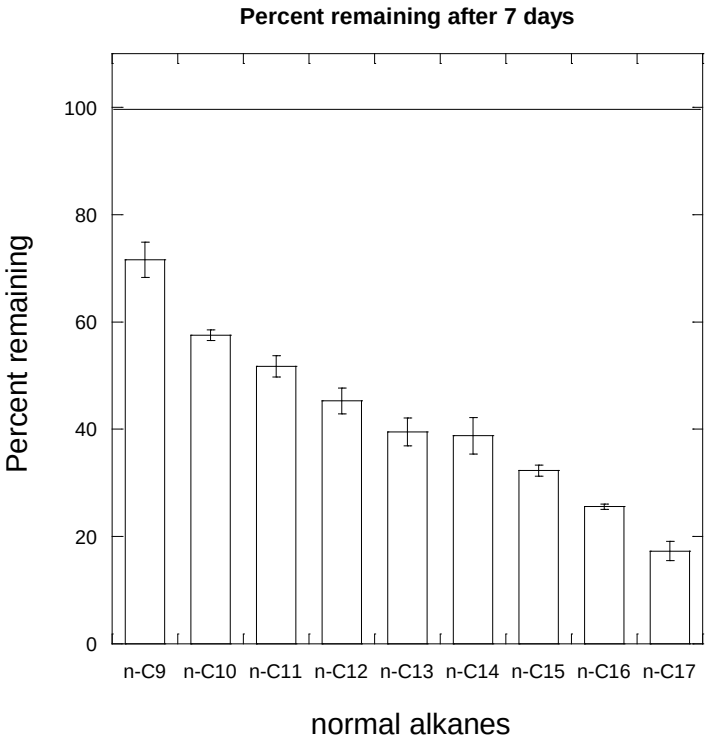


Figure 22. Normal alkanes consumed by *Yarrowia* exposed to Jet A.

Yarrowia was also tested in 1 Liter bottles (1:10 ratio experiments), with 100 mL of media, and 10 mL of Jet A and F76. These studies were performed for a period of 16 days for Jet A and 18 days for F76. Because we could harvest the fuel directly, there was no need to perform extractions, so samples were immediately examined using GCxGC. Only 30 mL of exposed sample and 15 mL of control fuel was obtained as a result of these experiments.

Results for the Jet A 1L bottle experiment showed the degradation of normal alkanes (**Table 20**); meanwhile, no degradation of isoparaffins, aromatics, or cycloalkanes was detected. The categories of hydrocarbon compounds that did not degrade did increase in concentration, as in other experiments because of the considerable loss of total n-alkanes from the mixture (originally 18.3% depleted to 15.8%). Because of the 3% absolute loss of exclusively n-alkanes, one would expect the concentration of the other compound classes in the mix to increase in

concentration. Fuels exposed in this way will have lower freeze points and generally better low temperature properties. Since only two bottles were examined using these protocol, we were unable to verify the likely result which was that the freeze point, pour point and cloud point could be changed by several degrees, depending on the size of the system involved. Similar results were obtained for the F76 experiment.

Table 20. GCxGC results for Jet A (4658) exposed to *Yarrowia lipolytica* in 1 liter bottles, for 16 days.

	Jet A (4658) in Yali d16 control 1a	Jet A (4658) in Yali d16 control 1b	Jet A (4658) in Yali d16 control 2	Jet A (4658) in Yali d16 exposed 1	Jet A (4658) in Yali d16 exposed 2
	Weight %	Weight %	Weight %	Weight %	Weight %
Aromatics					
Total Alkylbenzenes	**11.27**	**11.19**	**11.05**	**11.75**	**11.60**
Total Alkylnaphthalenes	**2.34**	**2.33**	**2.29**	**2.11**	**2.16**
Total Cycloaromatics	**6.75**	**6.75**	**6.76**	**6.45**	**6.64**
Total Aromatics	**20.36**	**20.27**	**20.10**	**20.31**	**20.40**
Paraffins					
Total iso-Paraffins	**32.60**	**32.67**	**32.71**	**33.79**	**33.68**
n-Paraffins					
n-C07	0.03	0.03	0.03	0.07	0.06
n-C08	0.18	0.18	0.17	0.31	0.27
n-C09	0.53	0.53	0.47	0.74	0.67
n-C10	1.51	1.50	1.38	1.86	1.72
n-C11	2.64	2.63	2.48	2.86	2.71
n-C12	3.36	3.36	3.27	3.14	3.07
n-C13	3.22	3.22	3.20	2.48	2.51
n-C14	2.74	2.81	2.84	2.01	2.07
n-C15	1.86	1.87	1.93	1.27	1.33
n-C16	0.96	1.00	1.09	0.62	0.66
n-C17	0.58	0.58	0.71	0.35	0.40
n-C18	0.18	0.18	0.26	0.08	0.10
n-C19	0.11	0.11	0.18	0.04	0.05
n-C20	0.08	0.09	0.14	0.03	0.04
n-C21	0.06	0.06	0.10	0.02	0.03
n-C22	0.04	0.04	0.06	0.01	0.02
n-C23	0.03	0.03	0.04	0.01	0.02
Total n-Paraffins	**18.11**	**18.21**	**18.33**	**15.89**	**15.71**
Cycloparaffins					
Total Monocycloparaffins	**22.75**	**22.63**	**22.88**	**23.67**	**23.82**
Total Dicycloparaffins	**6.14**	**6.17**	**5.98**	**6.31**	**6.34**
Total Tricycloparaffins	**0.04**	**0.04**	**<0.01**	**0.02**	**0.04**
Total Cycloparaffins	**28.93**	**28.84**	**28.86**	**30.01**	**30.20**

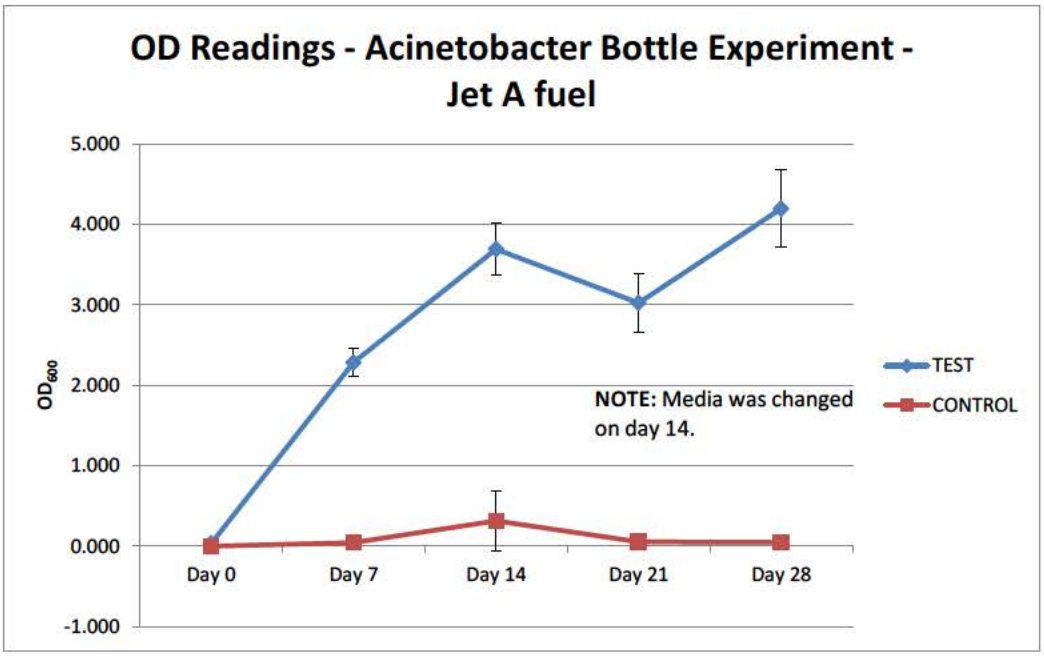
4.3.8 *Acinetobacter venetianus* in Jet A and F76 (DLA-08)

The experiments to date have shown that while bacteria consume different compounds, the bacteria being investigated have been most affecting the concentration of heavier n-alkanes (for *Pseudomonas* and *Acinetobacter*), light n-alkanes and n-alkylaromatics (*Marinobacter*), and all n-alkanes and select branched alkanes (*Yarrowia*). With the possible exception of thermal stability as measured by the Quartz Crystal Microbalance, no specification properties have been dramatically affected in fuel thus far, while some properties have been improved (freeze point, pour point). There may have been a slight decrease in thermal stability for F-76, but thermal stability is not a specification property of F-76.

In this portion of the research, we found through vial testing that the *Acinetobacter venetianus* forms an aggressive biofilm which emulsifies the fuel around it and could affect fuel properties (See **Figure 23**). It also grew to really high OD. Clearly, the formation of emulsified fuel and thick biofilms by the bacteria would affect the fuel delivery systems by plugging fuel filters and filter coalescers. It is extremely non-homogeneous in that it separates easily from the unaffected fuel.

Figure 23. Foam (emulsified fuel and biofilm) produced from a 28 day exposure of Jet A (left) to *Acinetobacter venetianus* or F76 (right).

The growth curves for the two *Acinetobacter* experiments in Jet A and F76 are shown in **Figure 24**. These are some of the highest OD values that have been collected with any of the experiments conducted for this program.



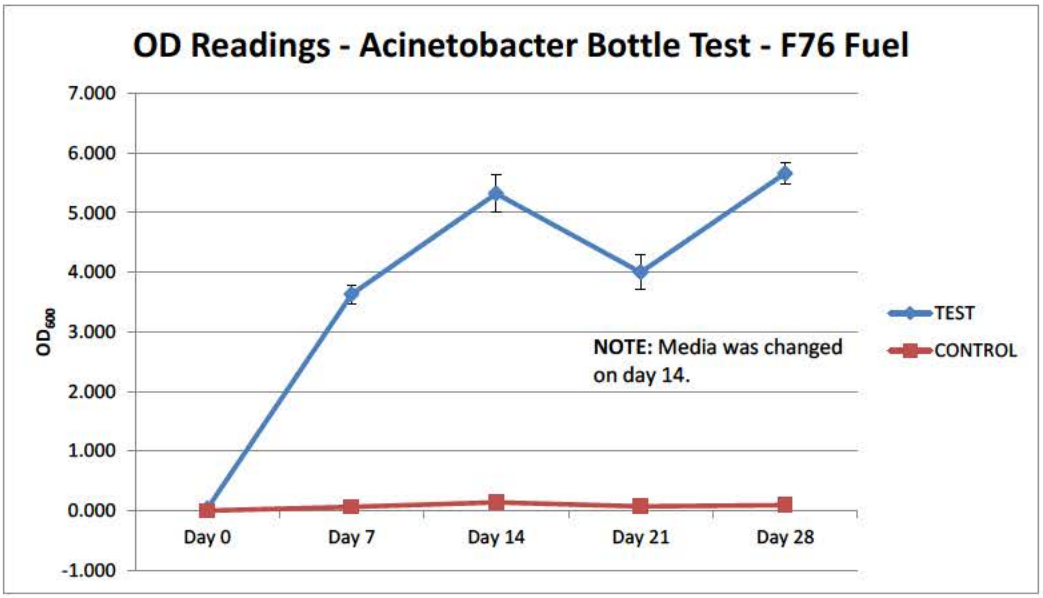


Figure 24. Growth curves for *Acinetobacter* in Jet A (top) and F76 (bottom) (DLA-08)

Acinetobacter in Jet A results are summarized below in **Table 21**. Note that, as predicted from the vial experiments, the *Acinetobacter* is primarily consuming n-alkanes, but to a lesser degree, some of the branched alkanes as well. F76 experimental results are provided in **Table**

22. In both experiments, *Acinetobacter* is creating significant degradation of the normal alkanes; in F76, the level of n-alkanes dropped from 15.3 to 10.8% (a 30% decrease in n alkanes). It would seem likely that all of the same issues concerning the reaction of n-alkanes in fuel would be similar to previous experiments: 1) aromatic levels (and other categories of compounds) would increase to make up for the loss of alkanes; 2) freeze points and other low temperature properties would be affected (improved) due to the decrease in the long chain n-alkanes; 3) all of the products of bacterial decay (alcohols , ketones and eventually carboxylic acids from the n-alkane degradation) would be present in low concentrations in the fuel. Unfortunately, it has been difficult to collect larger volumes of fuel from the *Acinetobacter* degradation due to the foam and emulsification of the fuel by the biofilm produced from this organism (**Figure 23**). Specification tests, which require more fuel, are difficult to perform because of the lack of fuel which is being emulsified in this powerful biofilm.

Table 21. *Acinetobacter* exposure to Jet A conducted for 28 days (DLA-08) .

	DLA-08 4658 acin control 09-25	DLA-08 4658 acinet exp 09-25
	Weight %	Weight %
Aromatics		
Total Alkylbenzenes	**11.26**	**11.08**
Total Alkylnaphthalenes	**1.86**	**1.84**
Total Cycloaromatics	**6.15**	**6.39**
Total Aromatics	**19.28**	**19.30**
Paraffins		
Total iso-Paraffins	**33.63**	**34.65**
n-Paraffins		
n-C07	0.03	0.06
n-C08	0.22	0.26
n-C09	0.63	0.66
n-C10	1.72	1.13
n-C11	2.90	1.90
n-C12	3.46	2.66
n-C13	3.16	2.34
n-C14	2.82	2.02
n-C15	1.85	1.31
n-C16	0.97	0.64
n-C17	0.48	0.36
n-C18	0.11	0.08
n-C19	0.05	0.04
n-C20	0.03	0.03
n-C21	0.02	0.02
n-C22	0.01	0.01
n-C23	<0.01	0.02
Total n-Paraffins	**18.47**	**13.51**
Cycloparaffins		
Total Monocycloparaffins	**22.93**	**25.35**
Total Dicycloparaffins	**5.66**	**7.14**
Total Tricycloparaffins	**0.02**	**0.05**
Total Cycloparaffins	**28.62**	**32.53**

Table 22. *Acinetobacter* exposure to F-76 conducted for 28 days (DLA-08)

	DLA-08 F76 acin control	DLA-08 acinet f76 exposed
	Weight %	Weight %
Aromatics		
Total Alkylbenzenes	**11.81**	**12.28**
Total Alkylnaphthalenes	**7.37**	**7.75**
Total Cycloaromatics	**7.63**	**7.98**
Total Triaromatics	**0.99**	**1.01**
Total Aromatics	**27.79**	**29.02**
Paraffins		
Total iso-Paraffins	**24.70**	**25.95**
n-Paraffins		
n-C07	<0.01	<0.01
n-C08	0.03	0.03
n-C09	0.15	0.13
n-C10	0.42	0.17
n-C11	0.67	<0.01
n-C12	0.71	0.40
n-C13	0.80	0.50
n-C14	1.10	0.81
n-C15	1.48	1.09
n-C16	1.79	1.35
n-C17	2.69	2.23
n-C18	1.47	1.06
n-C19	1.19	0.84
n-C20	1.02	0.77
n-C21	0.73	0.54
n-C22	0.48	0.36
n-C23	0.30	0.21
n-C24	0.15	0.12
n-C25	0.07	0.06
n-C26	0.04	0.03
n-C27	0.02	0.01
Total n-Paraffins	**15.31**	**10.75**
Cycloparaffins		
Total Monocycloparaffins	**24.34**	**25.71**
Total Dicycloparaffins	**7.83**	**8.54**
Total Tricycloparaffins	**0.03**	**0.03**
Total Cycloparaffins	**32.19**	**34.28**

4.3.9 Consortia Experiments: Jet A exposed to a mixture of *Acinetobacter* and *Pseudomonas* (DLA-09, completed 10-21-2013)

The purpose of these experiments was to compare the growth from two different organisms to the growth and degradation produced by the individual microorganisms. The consortia did not have as high a growth rate as measured by the OD reading (**Figure 25**) as the *Acinetobacter* or *Pseudomonas* itself (approximately 2.5), but the growth was still very high (OD reading of approximately 2.0). Comparison of the GCxGC analysis of the two experiments showed that the results of fuel decomposition are very much the same: about 5% of the fuel normal alkanes were lost due to decomposition (**Table 23**). Since both organisms are powerful normal alkane decomposers, this result is not surprising.

Figure 26 shows a plot generated from the GCxGC information on the consortia experiment. Samples were taken at the end point of the study only. The results indicate that the consortia consumed up to 30% of the original amount of the most useable hydrocarbons (mid-normal alkanes). There appeared to be some issues with the control samples in this experiment in that the C8-C10 compounds are lower in the day 28 controls than they should be. However, the controls for the less volatile compounds look reasonable. These results did not affect the concentrations of the mid-range normal alkanes and the microbes' ability to consume these compounds.

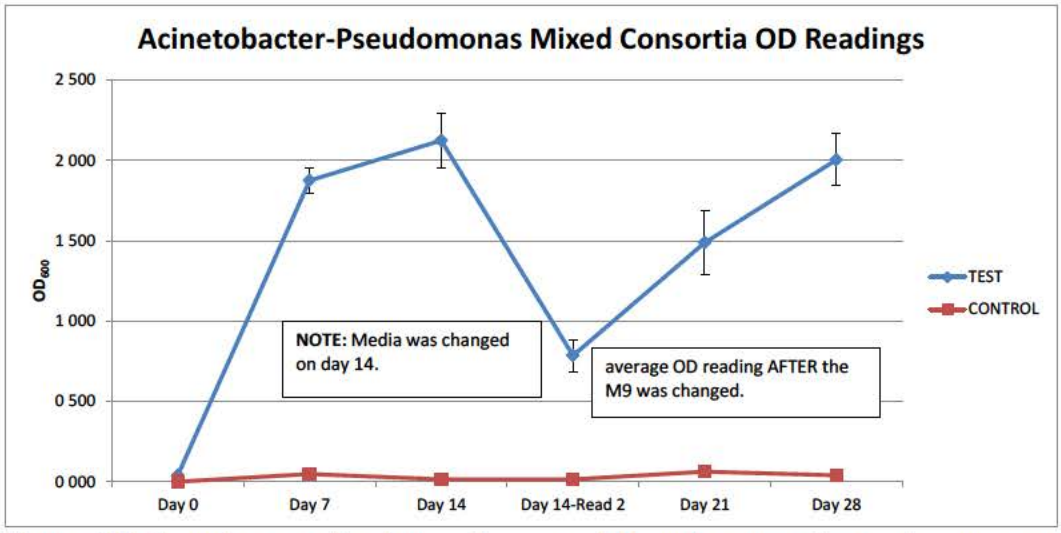


Figure 25. Growth curve for *Acinetobacter* and *Pseudomonas* Consortia exposure to Jet A conducted for 28 days (DLA-09)

Table 23. *Acinetobacter* and *Pseudomonas* Consortia exposure to Jet A conducted for 28 days (DLA-09)

	neat 4658-filt 10-24	consortia 4658 control 10-21	consortia 4658 exp 10-21
	Weight %	Weight %	Weight %
Aromatics			
Total Alkylbenzenes	**13.55**	**13.45**	**14.08**
Total Alkylnaphthalenes	**1.85**	**1.89**	**2.00**
Total Cycloaromatics	**5.81**	**5.95**	**6.30**
Total Aromatics	**21.20**	**21.28**	**22.38**
Paraffins			
Total iso-Paraffins	**31.04**	**30.86**	**32.60**
n-Paraffins			
n-C07	0.19	0.04	0.10
n-C08	0.53	0.36	0.40
n-C09	1.12	1.01	1.00
n-C10	2.52	2.51	2.13
n-C11	3.61	3.78	3.08
n-C12	3.71	3.84	2.77
n-C13	2.86	2.98	1.77
n-C14	2.10	2.22	1.64
n-C15	1.27	1.31	0.99
n-C16	0.59	0.63	0.45
n-C17	0.23	0.28	0.17
n-C18	0.05	0.05	0.04
n-C19	0.02	0.02	0.01
n-C20	<0.01	<0.01	<0.01
n-C21	<0.01	<0.01	<0.01
n-C22	<0.01	<0.01	<0.01
n-C23	<0.01	<0.01	<0.01
Total n-Paraffins	**18.83**	**19.03**	**14.57**
Total Monocycloparaffins	**23.00**	**22.33**	**24.01**
Total Dicycloparaffins	**5.80**	**6.39**	**6.28**
Total Tricycloparaffins	**0.13**	**0.11**	**0.15**
Total Cycloparaffins	**28.93**	**28.83**	**30.44**

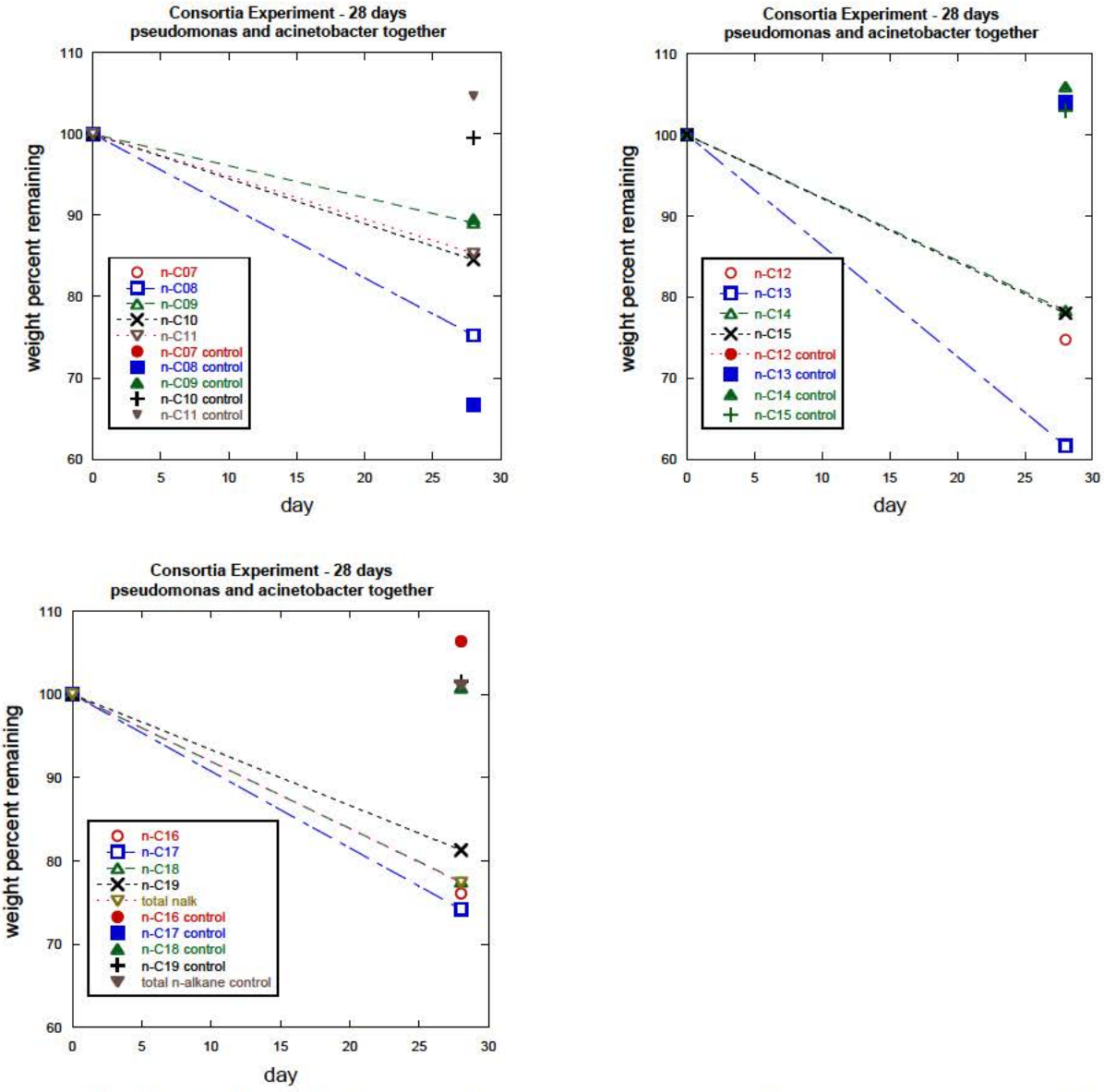


Figure 26. Graphical results from combined experiment where the two organisms are added to the media at the beginning of the test. Results show that the degradation rate of 20-40% for the heavier normal alkanes is similar for sequential or combined *Pseudomonas*/*Acinetobacter* exposure. Control sample issues with C7-C9 were also observed due to evaporation.

4.3.10 Sequential Experiments: *Acinetobacter* with *Pseudomonas* Mixed Consortia exposed to Jet A (DLA-10, completed 10-24-14)

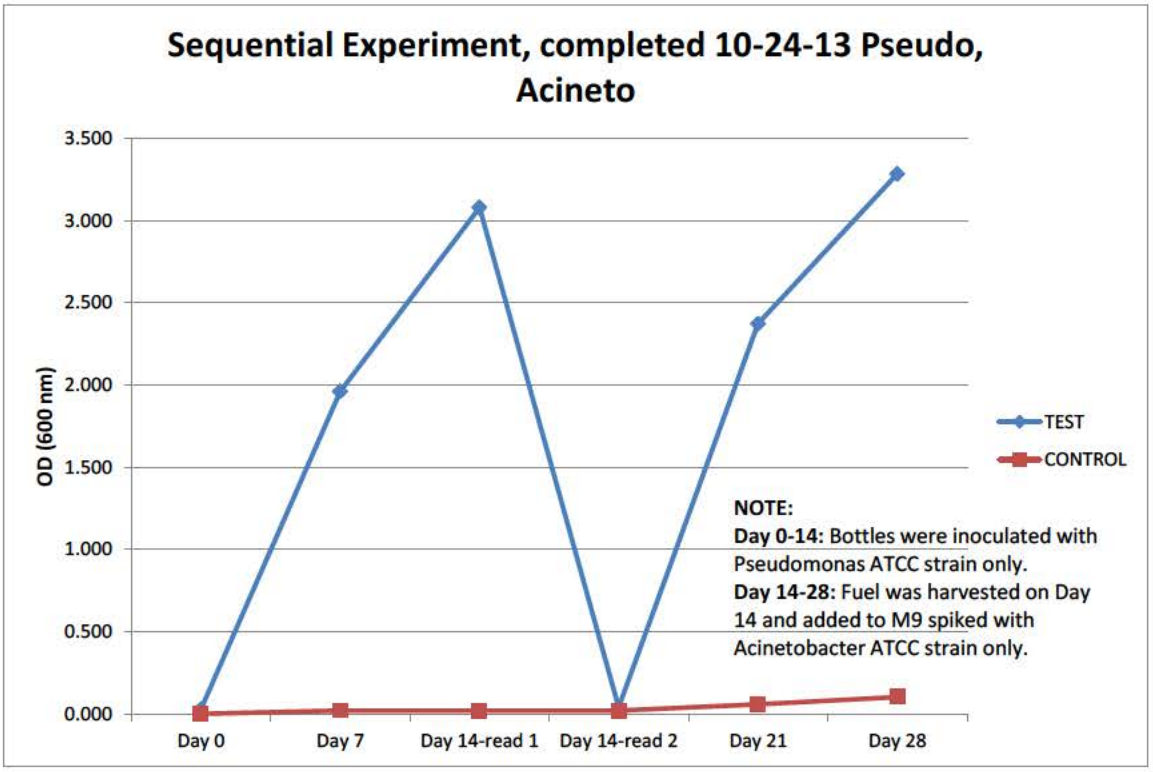


Figure 27. Sequential experiment for *Pseudomonas* (14 days) followed by *Acinetobacter* (14 days).

Pure bacterial cultures may show enormous growth in fuel:aqueous mixtures, since there may be no competition for resources from other bacteria. In these experiments, we performed testing in 1 Liter bottles to determine whether using a pure culture of bacteria showed more degradation than the sequential exposure of two types of bacteria in a similar time period. The pure culture experiment (DLA-09) was described above for the consortia of *Pseudomonas* and *Acinetobacter*. In this experiment, DLA-10, *Pseudomonas aeruginosa* was exposed for the first two weeks using 11 one-liter Teflon bottles with 100 mL of aqueous media, and 10 mL of fuel (Jet A, 4658). In order to keep oxygen in the system, the bottles were uncapped for several

minutes every week and air allowed to replenish the headspace of the bottle. At the two week point, three bottles were sampled by withdrawing less than 1 mL of fuel, and the samples analyzed using GCxGC.

After the 15 day exposure of *Pseudomonas*, the fuel was collected and centrifuged to separate it from the water and bacteria, and this fuel then, was reintroduced into the second part of the experiment with *Acinetobacter venetianus* exposure (see **Figure 27**). In this experiment, the *Acinetobacter* consumed an additional 1.4% (absolute) of the normal alkanes which was a slightly lower rate than the *Pseudomonas* (See **Figure 28**). The percentage of the n-alkanes consumed by *Pseudomonas* was 11.3% (2.12% consumed, 18.82% available) while the *Acinetobacter* consumed 8.4% (1.4% consumed, 16.70% available). Very few other changes in the chemical composition of the fuel samples occurred due to the sequential exposure of the bacteria (**Table 24**).

The results of the combined bacteria are shown in **Figure 28** for the same time period. The n-alkanes were reduced 4.5% absolute in the combined experiment as compared to 3.5% for the sequential experiment. These results are somewhat similar in the sense that the sequential experiment removed a thriving population of *Pseudomonas* from an existing system. These were replaced with a lower concentration of *Acinetobacter* and this lower concentration would certainly take time to become established and consume as much *n*-alkane as the established *Pseudomonas*.

Overall then**, the consumption of total n-alkanes was similar in the sequential experiment as in the combined experiment.** These two bacteria, *Pseudomonas* and *Acinetobacter* seem very similar to each other in the sense that they consume the n-alkanes

preferentially, and they are both fast growing species when jet fuel, with high concentrations of alkanes, is present. In this case, there was no evidence that they interfered with each other in growth rates, or in any way enhanced the growth of one another due to a preference for consuming different fuel components. The only obvious difference between these two bacteria is the formation of the more aggressive biofilm in the case of the *Acinetobacter*.

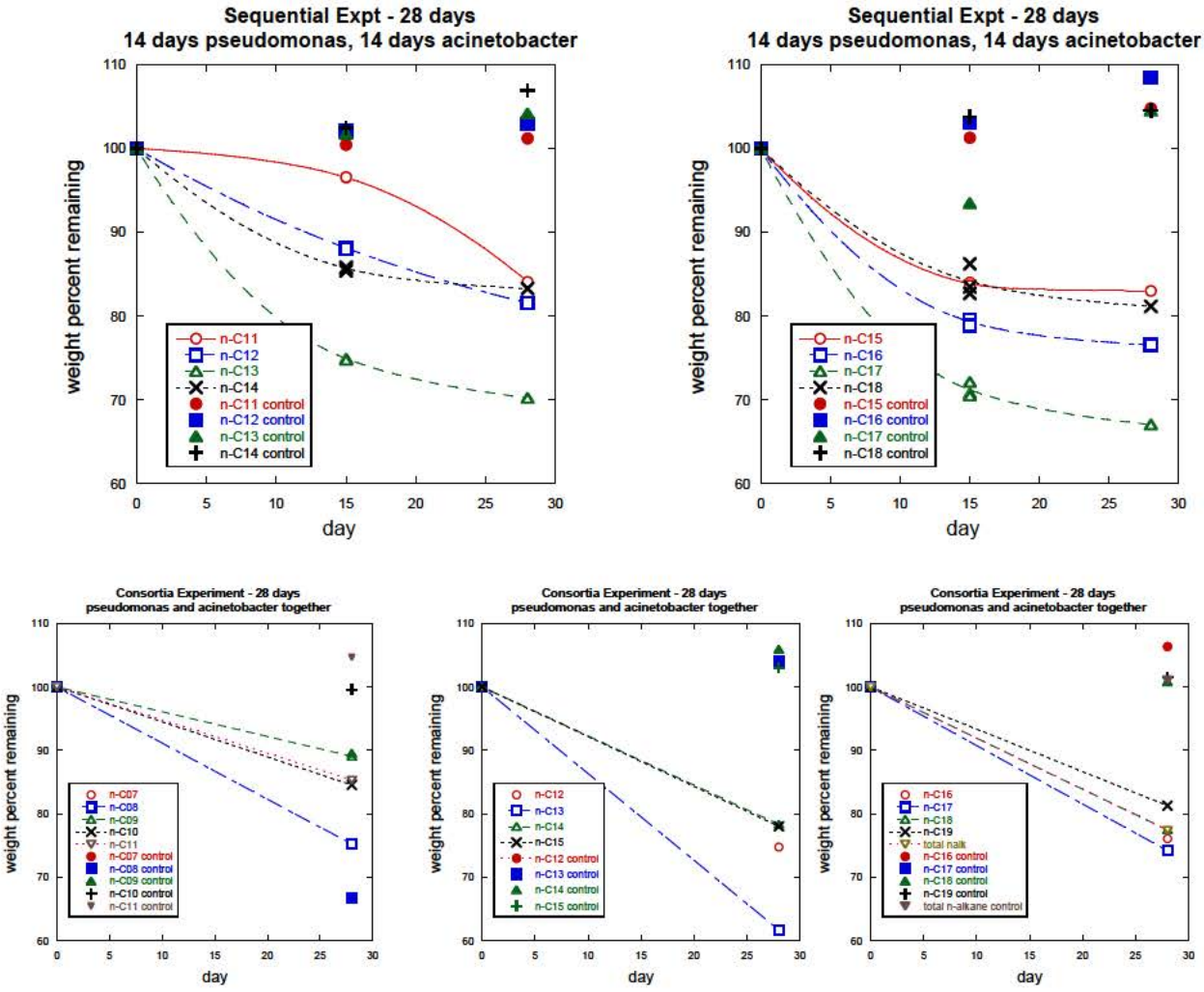


Figure 28. (Top) Graphical results from sequential experiment. Results show slightly faster rate of degradation of heavier normal alkanes with the *Pseudomonas*, as compared to the second 14 day period with *Acinetobacter*. (Bottom) Consortia experiment plots taken from **Figure 26** show similar total degradation at the 28 day end point.

Table 24. Sequential experiment for *Pseudomonas* (14 days) followed by *Acinetobacter* (14 days) (DLA-10)

	neat 4658 filt 10-24	seq con1 d14 10-10	seq con10 d14 10-10	seq con15 d14 10-10	seq con d14 Ave.	seq con d14 Stdev	seq con d14 %RSD	seq exp1 d14 10-10	seq exp2 d14 10-10	seq exp3 d14 10-10	seq exp d14 Ave.	seq exp d14 Stdev	seq exp d14 %RSD	seq con d28 10-24	seq exp d28 10-24
	Weight %	Weight %	Weight %	Weight %	Weight %			Weight %	Weight %	Weight %	Weight %			Weight %	Weight %
Aromatics															
Total Alkylbenzenes	**13.55**	**12.43**	**12.43**	**12.45**	**12.44**	**0.01**	**0.1**	**12.75**	**12.78**	**12.79**	**12.77**	**0.02**	**0.2**	**11.40**	**11.93**
Total Alkylnaphthalenes	**1.86**	**1.84**	**1.82**	**1.81**	**1.82**	**0.01**	**0.7**	**1.86**	**1.87**	**1.85**	**1.86**	**0.01**	**0.6**	**1.80**	**1.87**
Total Cycloaromatics	**5.92**	**5.83**	**5.78**	**5.79**	**5.80**	**0.03**	**0.5**	**5.92**	**5.99**	**5.95**	**5.95**	**0.04**	**0.6**	**5.99**	**6.26**
Total Aromatics	**21.33**	**20.10**	**20.03**	**20.05**	**20.06**	**0.03**	**0.2**	**20.53**	**20.64**	**20.58**	**20.58**	**0.05**	**0.3**	**19.20**	**20.05**
Paraffins															
Total iso-Paraffins	**31.00**	**32.16**	**32.29**	**32.20**	**32.22**	**0.06**	**0.2**	**33.48**	**33.34**	**33.49**	**33.44**	**0.08**	**0.3**	**33.65**	**35.39**
n-Paraffins															
n-C07	0 15	0 06	0 08	0 08	0 07	0 01	15 2	0 09	0 08	0 09	0 09	0 00	5 3	0 02	0 05
n-C08	0 53	0 34	0 37	0 38	0 36	0 02	6 2	0 38	0 37	0 38	0 38	0 01	2 3	0 18	0 26
n-C09	1 12	0 88	0 91	0 91	0 90	0 02	2 0	0 92	0 91	0 92	0 92	0 01	0 8	0 63	0 72
n-C10	2 52	2 23	2 24	2 24	2 24	0 01	0 3	2 22	2 23	2 24	2 23	0 01	0 4	1 83	1 59
n-C11	3 61	3 50	3 48	3 48	3 49	0 01	0 3	3 29	3 32	3 31	3 31	0 01	0 4	3 09	2 49
n-C12	3 71	3 76	3 73	3 74	3 75	0 02	0 4	3 10	3 14	3 11	3 12	0 02	0 6	3 67	2 73
n-C13	2 86	3 12	3 09	3 10	3 10	0 02	0 5	2 19	2 23	2 19	2 20	0 02	0 9	3 24	2 02
n-C14	2 10	2 48	2 51	2 45	2 48	0 03	1 2	2 02	2 05	2 03	2 03	0 02	1 0	2 78	1 93
n-C15	1 27	1 51	1 50	1 50	1 50	0 01	0 5	1 24	1 25	1 20	1 23	0 02	1 9	1 76	1 24
n-C16	0 59	0 75	0 75	0 74	0 75	0 00	0 6	0 56	0 57	0 56	0 56	0 00	0 4	0 87	0 58
n-C17	0 27	0 33	0 29	0 33	0 32	0 02	7 7	0 29	0 27	0 28	0 28	0 01	2 5	0 42	0 36
n-C18	0 05	0 07	0 07	0 07	0 07	0 00	1 8	0 09	0 08	0 08	0 08	0 01	6 8	0 09	0 09
n-C19	0 02	0 02	0 02	0 02	0 02	0 00	2 4	0 02	0 02	0 02	0 02	0 00	0 7	0 03	0 02
n-C20	<0 01	<0 01	<0 01	<0 01	<0 01	NA	NA	<0 01	<0 01	<0 01	<0 01	NA	NA	0 01	0 01
n-C21	<0 01	<0 01	<0 01	<0 01	<0 01	NA	NA	<0 01	<0 01	<0 01	<0 01	NA	NA	<0 01	<0 01
n-C22	<0 01	<0 01	<0 01	<0 01	<0 01	NA	NA	<0 01	<0 01	<0 01	<0 01	NA	NA	<0 01	<0 01
n-C23	<0 01	<0 01	<0 01	<0 01	<0 01	NA	NA	<0 01	<0 01	<0 01	<0 01	NA	NA	<0 01	<0 01
Total n-Paraffins	**18.82**	**19.06**	**19.05**	**19.05**	**19.05**	**0.00**	**0.0**	**16.44**	**16.53**	**16.45**	**16.47**	**0.05**	**0.3**	**18.64**	**14.10**
Cycloparaffins															
Total Monocycloparaffins	**22.78**	**22.62**	**22.79**	**22.68**	**22.70**	**0.08**	**0.4**	**23.28**	**23.18**	**23.06**	**23.18**	**0.11**	**0.5**	**22.80**	**24.38**
Total Dicycloparaffins	**6.02**	**5.96**	**5.76**	**5.91**	**5.88**	**0.11**	**1.8**	**6.17**	**6.21**	**6.31**	**6.23**	**0.07**	**1.1**	**5.67**	**6.04**
Total Tricycloparaffins	**0.05**	**0.10**	**0.08**	**0.10**	**0.09**	**0.01**	**10.2**	**0.10**	**0.10**	**0.10**	**0.10**	**0.00**	**0.0**	**0.05**	**0.05**
Total Cycloparaffins	**28.85**	**28.68**	**28.63**	**28.69**	**28.67**	**0.04**	**0.1**	**29.56**	**29.49**	**29.47**	**29.51**	**0.04**	**0.1**	**28.51**	**30.46**

4.3.11 Exposure of *Acinetobacter* to Jet A (DLA-11, 10-25-13)

Figure 29 shows the growth curves for this replicate experiment; the growth curves are very similar to DLA-08. These experiments are being repeated because the amount of fuel collected in order to conduct specification testing is so small. Less than half of the original fuel is collected from these tests. As the experiment was conducted, we considered this loss to be experimental; leaking bottles, or evaporation. As we started to understand this microbe and the foam that it produces, we started to consider that the fuel was being incorporated into this foam emulsion and therefore could not be tested using large amounts of fuel, even if the foam was centrifuged. The GCxGC experiments were conducted like the other experiments, however (**Table 25**).

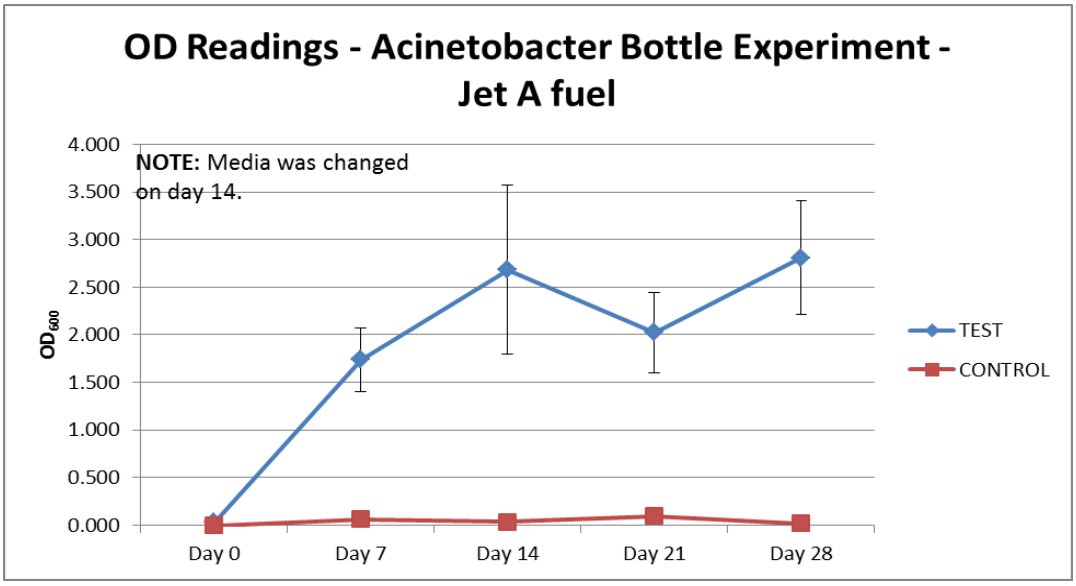


Figure 29. *Acinetobacter* exposure to Jet A, conducted as a replicate of DLA-08. (DLA-11) Completed on 10-25-13.

The foam will be problematic to the fuel quality, obviously. A specification test was not needed to see that the material will not flow easily and would not pump easily. There was no way to filter this material either. But, only small amounts of this bio-foam would be in fuel from a tank. There may be instances where a small water-bottoms, containing *Acinetobacter* foam may be swept up into the fuel and small amounts of foam could be subjected to use on-board. Therefore, an experiment was conceived where a small amount of foam (500 ppm volume or 0.05%) was mixed vigorously by mechanical shaking for 30 minutes into Jet A fuel and the mixture tested for specification test. Essentially, this small contamination would simulate what might happen in a tank with a small amount of *Acinetobacter* contamination.

The results from specification testing are provided in **Table 26**. There is a comparison to the neat fuel provided in the table. The only difference of any significance is the increase in the particulate matter filtration time, which increased from 4 minutes to 7 minutes as the foam was added. There was no increase in the amount of particulate captured in the filtration, but the increase in filtration time would indicate that the foam may have plugged filter pores, as would be expected. At 500 ppm, the concentration of foam is not sufficient to register a weight increase for the particulate. There were few other changes that were meaningful with the exception of JFTOT, which did not fail with the addition of the additive, but increased from a tube rating of 1 to a tube rating of 2. While this change is not definitive, it does indicate that the foam would, as expected, make the fuel thermally unstable.

The lubricity of the sample did not change significantly due to the foam. The Jet A, POSF-4658 had a wear scar (lubricity) of 0.71 mm when the fuel was measured directly. If the fuel is subjected to media in a control-type experiment, that wear scar will decrease (lubricity improves) to 0.57 mm, which occurred in the carboy experiments (DLA-04). If the fuel is

exposed to *Pseudomonas*, as in the carboy experiment, then the wear scar drops even further to a level of 0.41. We have not observed an increase in wear scar diameter yet in the Jet A, but this Jet A does not contain corrosion inhibitor, which other authors have shown is consumed by bacteria.

Table 25. *Acinetobacter* data completed 12-5-13 (DLA-11))

	neat 4658 filt 10-24	acinetobacter 4658 d14 con1	acinetobacter 4658 d14 con5	acinetobacter 4658 d14 con 15	acinetobacter 4658 d14 exp 5	acinetobacter 4658 d14 exp10	acinetobacter 4658 d14 exp 15	acinetobacter 4658 d28 Con	acinetobacter 4658 d28 Exp
	Weight %	Weight %	Weight %	Weight %	Weight %	Weight %	Weight %	Weight %	Weight %
Aromatics									
Total Alkylbenzenes	13.55	12.41	12.43	12.43	12.74	11.79	11.78	11.38	11.15
Total Alkylnaphthalenes	1.86	1.85	1.82	1.84	1.82	1.81	1.78	1.79	1.70
Total Cycloaromatics	5.92	5.90	5.82	5.83	5.92	5.88	5.81	6.02	5.92
Total Aromatics	21.33	20.16	20.07	20.10	20.49	19.48	19.37	19.19	18.77
Paraffins									
Total iso-Paraffins	31.00	32.27	32.43	32.42	32.71	34.56	34.85	33.42	35.57
n-Paraffins									
n-C07	0.15	0 07	0.07	0.08	0.09	0.07	0.08	0.03	0.06
n-C08	0.53	0 34	0.36	0.36	0.39	0.32	0.34	0.21	0.29
n-C09	1.12	0 87	0.89	0.88	0.92	0.77	0.80	0.65	0.74
n-C10	2.52	2 21	2.23	2.21	2.01	1.71	1.74	1.85	1.38
n-C11	3.61	3.47	3.47	3.46	3.01	2.81	2.79	3.11	2.20
n-C12	3.71	3.76	3.74	3.74	3.44	3.30	3.25	3.66	2.90
n-C13	2.86	3.10	3.07	3.12	2.79	2.87	2.80	3.26	2.49
n-C14	2.10	2 55	2.47	2.53	2.24	2.40	2.36	2.78	2.11
n-C15	1.27	1 52	1.51	1.51	1.37	1.58	1.56	1.77	1.40
n-C16	0.59	0.73	0.75	0.75	0.65	0.80	0.82	0.87	0.71
n-C17	0.27	0 29	0.34	0.29	0.26	0.63	0.74	0.42	0.51
n-C18	0.05	0 07	0.07	0.07	0.06	0.21	0.29	0.09	0.17
n-C19	0.02	0 02	0.02	0.02	0.02	0.02	0.02	0.03	0.02
n-C20	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01	0.01	0.01
n-C21	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
n-C22	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
n-C23	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Total n-Paraffins	18.82	19.01	19.02	19.05	17.26	17.53	17.61	18.75	15.02
Cycloparaffins									
Total Monocycloparaffins	22.78	22.51	22.41	22.35	23.14	22.27	22.09	22.84	24.31
Total Dicycloparaffins	6.02	5.95	5.96	5.97	6.29	6.07	5.99	5.72	6.28
Total Tricycloparaffins	0.05	0.10	0.10	0.10	0.10	0.10	0.10	0.09	0.04
Total Cycloparaffins	28.85	28.57	28.47	28.43	29.54	28.43	28.17	28.64	30.64

Table 26. Jet A containing 500 ppmv *Acinetobacter*-produced foam biofilm was tested by specification tests for Jet A (DLA-11)

ASTM Test #	SPECIFICATION TESTS	Jet A spec min	Jet A spec max	POSF-4658 7/11/2012	500 ppmv foam in Jet A (4658) 12/12/2013
	Workmanship		pass		pass
D3242	Total Acid Num. (mg KOH/g)		0.1	0.002	0.004
D1319	Aromatics, vol %		25	19	20
D3227	Mercaptan Sulfur, wt %		0.003	0.000	0.000
D4294,1266,1552,2622	Total Sulfur, wt%		0.3	0.00	0.04
D86 or D2887	Distillation D86 or D2887				
	IBP, deg C		Report		
	10% recovered, deg C		205	180	183
	20% recovered, deg C		Report		191
	50% recovered, deg C		Report	212	213
	90% recovered, deg C		Report	251	252
	EF, deg C		300	274	278
	Residue, vol%		1.5	1.3	1.2
	loss, vol%		1.5	0.9	0.7
D93	Flash point, degrees C	38		51	52
D5972	Freeze Point, degrees C		-40	-48	-47
D445	Viscosity @ -20, cSt		8	5.2	5.1
D4809	Heat of Comb. (calc), MJ/kg			43.2	43.2
D1322	Smoke Point, mm	18		21	23
D1840	Naphthalenes, vol %		3		
D130	Copper Strip Corrosion		1	1a	1a
D3241	Thermal Stability @ 260°C				
	Tube Deposit Rating		<3	1	2
	Change in Pressure, mmHg		25	0	0
D381	Existent Gum, mg/100mL		7	0.2-2.0	<1
D5452	Particulate Matter, mg/L		1	1.2	1.3
	Filtration Time, minutes		15	4	7
D1094	Water Reaction		1B	1	1
D5006	FSII, vol%	none	none	ND	ND
D2624	Conductivity, pS/m	50	450	ND	ND
D4052	API Gravity @ 60 F	37	51		
D4052	specific gravity, g/mL	0.84	0.775	0.806	0.807
D5001	Lubricity	report	report	0.71	0.69

4.3.12 *Acinetobacter* exposure to Jet A (DLA-12, 12-5-13).

This experiment was again replicated due to poor recoveries, likely related to the formation of the foam material, as discussed above. The growth curves for this experiment are shown in **Figure 30** for the 27 day test conducted. Results for growth are similar to previous results provided. **Table 27** shows the GCxGC results for this additional *Acinetobacter* test. There is once again a 4% loss of the normal alkanes, and no other significant additional changes other than the fact that the foam emulsification occurred.

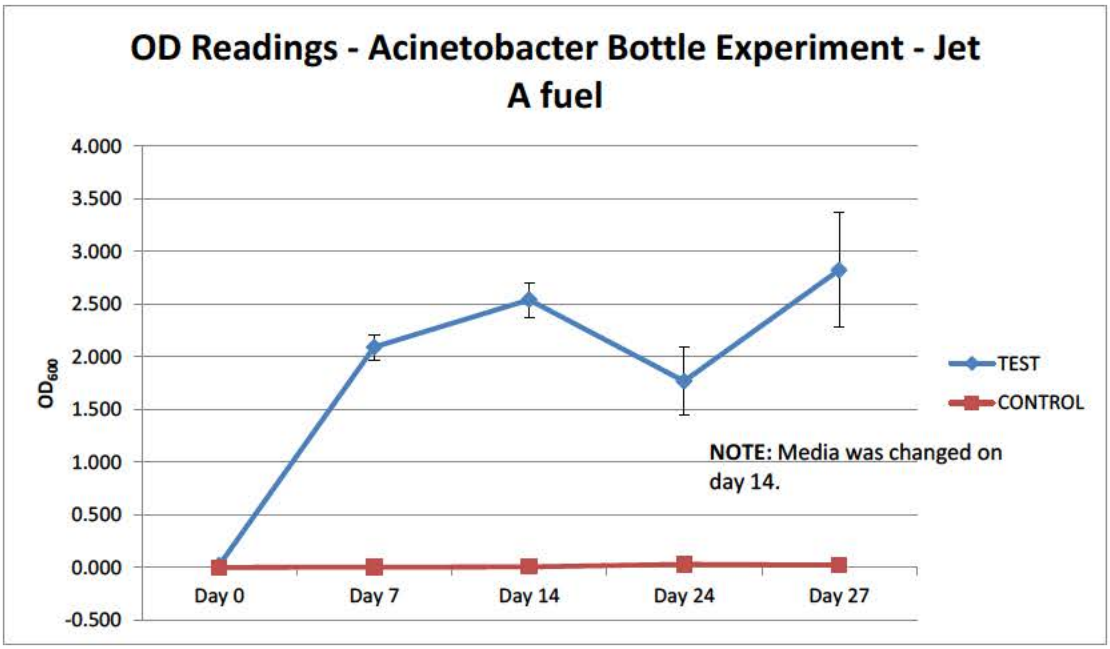


Figure 30. *Acinetobacter* exposure to Jet A, conducted as another replicate of DLA-08 and DLA-11). (DLA-12) Completed on 12-5-13.

Table 27. *Acinetobacter* exposure to Jet A for 27 days, ending 12-5-13 (DLA-12)

	control 4658 d28	acineto rep a,d27	Acineto,rep b, d27 (4658)	acineto d 27 rep c 12-5-13
	Weight %	Weight %	Weight %	Weight %
Aromatics				
Total Alkylbenzenes	**13.50**	**14.23**	**14.19**	**14.16**
Total Alkylnaphthalenes	**1.89**	**1.96**	**1.99**	**1.98**
Total Cycloaromatics	**5.88**	**6.04**	**6.10**	**6.08**
Total Aromatics	**21.27**	**22.24**	**22.28**	**22.22**
Paraffins				
Total iso-Paraffins	**30.93**	**31.39**	**31.79**	**31.71**
n-C07 & lower	0.07	0.11	0.12	0.12
n-C08	0.40	0.43	0.43	0.43
n-C09	1.03	0.99	0.99	0.99
n-C10	2.51	1.78	1.78	1.78
n-C11	3.65	2.65	2.65	2.64
n-C12	3.82	3.12	3.11	3.12
n-C13	2.98	2.52	2.53	2.52
n-C14	2.24	1.94	1.94	1.94
n-C15	1.33	1.19	1.18	1.18
n-C16	0.64	0.56	0.56	0.56
n-C17	0.28	0.22	0.22	0.22
n-C18	0.06	0.05	0.05	0.05
n-C19	0.02	0.02	0.01	0.01
n-C20	<0.01	<0.01	<0.01	<0.01
n-C21	<0.01	<0.01	<0.01	<0.01
n-C22	<0.01	<0.01	<0.01	<0.01
n-C23	<0.01	<0.01	<0.01	<0.01
Total n-Paraffins	**19.05**	**15.60**	**15.58**	**15.58**
Cycloparaffins				
Total Monocycloparaffins	**22.48**	**24.28**	**23.79**	**23.99**
Total Dicycloparaffins	**6.17**	**6.39**	**6.45**	**6.38**
Total Tricycloparaffins	**0.10**	**0.11**	**0.11**	**0.11**
Total Cycloparaffins	**28.75**	**30.77**	**30.34**	**30.48**

4.3.13 Sequential Experiments: *Acinetobacter* with *Pseudomonas* Mixed Consortia exposed to Jet A (DLA-13, 12-23-13).

This experiment was conducted to be a replicated of the DLA-10 experiment and it showed very similar growth (**Figure 31**). In these experiments, the GCxGC results showed again that about 2% loss of the normal alkanes occurred for each of the bacteria separately, adding up to a total of almost 4% loss of the same mid-range normal alkanes (**Table 28**).

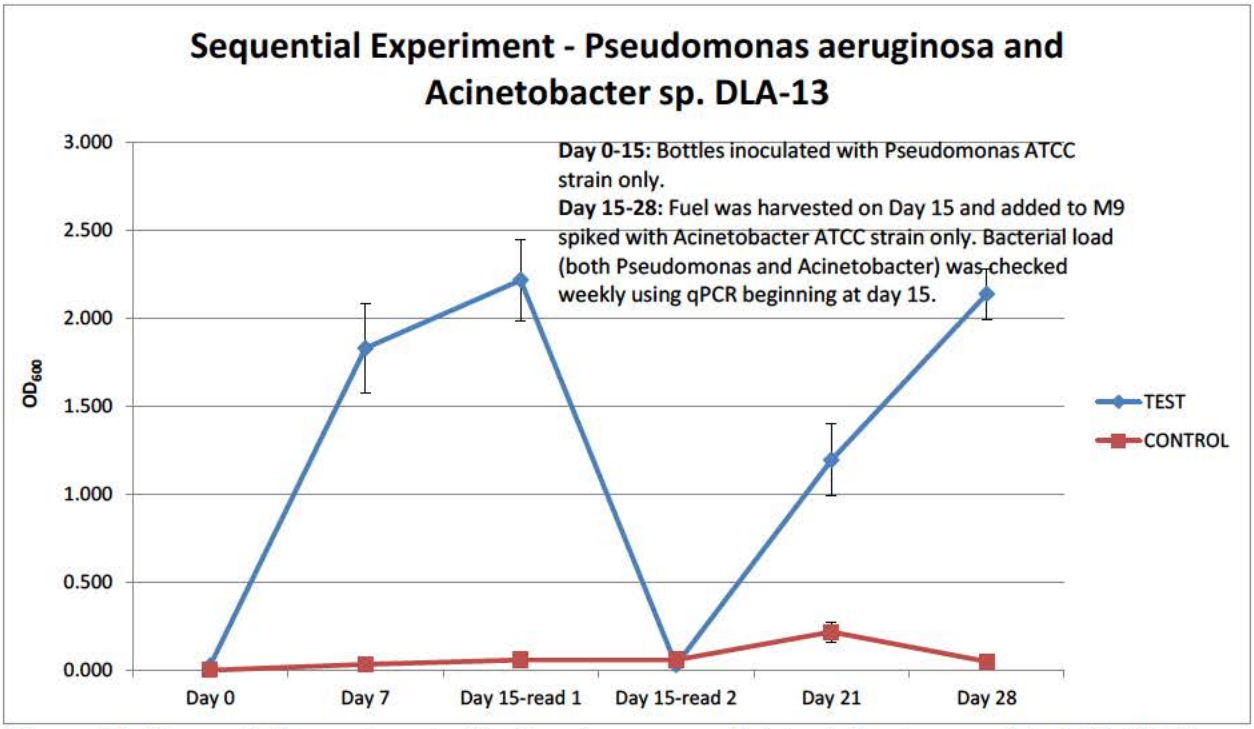


Figure 31. Sequential experiment with *Pseudomonas* and *Acinetobacter* completed 12-23-13 (DLA-13)

Table 28. GCxGC results for sequential experiment with *Pseudomonas* and *Acinetobacter* completed 12-23-13 (DLA-13)

	neat 4658-filt 10-24	Seq pseudod 15a	Seq pseudo d15b	Seq pseudo d15 c	Seq control d15	seq pseudo acineto d28	seq d28 control
	Weight %	Weight %	Weight %	Weight %	Weight %	Weight %	Weight %
Aromatics							
Total Alkylbenzenes	13.55	13.94	13.97	13.96	13.61	14.15	13.50
Total Alkylnaphthalenes	1.86	1.91	1.91	1.90	1.88	1.96	1.89
Total Cycloaromatics	5.92	5.90	5.88	5.90	5.75	6.14	5.88
Total Aromatics	21.33	21.75	21.76	21.77	21.25	22.26	21.27
Paraffins							
Total iso-Paraffins	31.00	31.75	31.94	31.86	30.78	32.07	30.93
n-Paraffins							
n-C07	0.15	0.14	0.14	0.17	0.13	0.10	0.07
n-C08	0.53	0.48	0.48	0.48	0.46	0.42	0.40
n-C09	1.12	1.09	1.09	1.10	1.07	1.02	1.03
n-C10	2.52	2.49	2.49	2.49	2.50	2.15	2.51
n-C11	3.61	3.48	3.48	3.48	3.62	3.03	3.65
n-C12	3.71	3.27	3.27	3.27	3.79	3.03	3.82
n-C13	2.86	2.15	2.15	2.14	2.91	2.01	2.98
n-C14	2.10	1.80	1.80	1.79	2.15	1.75	2.24
n-C15	1.27	1.07	1.06	1.06	1.28	1.05	1.33
n-C16	0.59	0.47	0.47	0.47	0.61	0.45	0.64
n-C17	0.27	0.19	0.19	0.19	0.25	0.23	0.28
n-C18	0.05	0.05	0.05	0.04	0.06	0.04	0.06
n-C19	0.02	0.01	0.01	0.01	0.02	0.01	0.02
n-C20	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
n-C21	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
n-C22	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
n-C23	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Total n-Paraffins	18.82	16.71	16.69	16.72	18.87	15.32	19.05
Cycloparaffins							
Total Monocycloparaffins	22.78	23.50	23.29	23.37	23.00	23.89	22.48
Total Dicycloparaffins	6.02	6.19	6.22	6.18	6.01	6.35	6.17
Total Tricycloparaffins	0.05	0.11	0.10	0.11	0.10	0.11	0.10
Total Cycloparaffins	28.85	29.80	29.61	29.66	29.11	30.35	28.75

4.4 Additives in Jet Fuel and microbial degradation

Over the previous 9 months of this project, we have worked toward investigating whether microbes could consume enough specific fuel components to change the properties of the fuel. Because certain bacteria are targeting specific compounds within the fuel, and composition of jet fuel is directly related to physical and chemical properties, it is prudent to determine whether fuel properties could be affected in this way. So far, we have observed small changes in the low temperature properties (freeze point, pour point and cloud point) of some fuels when bacteria (like some *Pseudomonas* species) consume heavier (> ~C14) normal alkanes. This testing has only shown extremely small changes in acid number of fuel; one would suspect that bacterial degradation would create changes in the acidity of fuel, but these increases are difficult to measure or project. The only other properties that change significantly are the ones one would expect to change if an insoluble foam were to become incorporated into the fuel being filtered or heated. So, while fuel properties or hydrocarbon composition does not appear to be affected in any significant way other than these, we wanted to examine the possibility that bacteria may consume additives which give specific properties to the fuel.

The additives in fuel that were examined were antioxidants, corrosion inhibitors and the fuel system icing inhibitor, diethylene glycol monomethylether (DiEGME).

4.4.1 Antioxidants

Antioxidants are not required additives, but are often added at the refinery to slow oxidation in storage or under thermal stress conditions. Often, a severely hydrotreated fuel loses the natural antioxidants it contained (nitrogen-, sulfur- and oxygen-containing compounds) and these need to be replaced by antioxidants such as substituted phenols. The most common

antioxidant for fuel, butylated hydroxytoluene (BHT) was studied in these experiments. Our experiments showed that exposing fuel with BHT to bacteria did not lead to the degradation of BHT. A possible explanation for this is that BHT 1) is both highly non-polar, which prevented it being solubilized into the water phase where microbes reside, 2) is recalcitrant to degradation due to the toxic nature of its aromatic ring and side chains, or 3) simply is not consumed by these bacteria because these bacteria consume n-alkanes.

The antioxidant, BHT was added to Jet A fuel at a concentration of 25 mg/L, which is a typical level for jet fuel. *Pseudomonas* ATCC bacterium was cultivated for 24 hours in Lysogeny broth (LB). A conical tube was centrifuged to pellet out the cultured cells. The LB broth was decanted and the tube was washed twice with M-9 minimal media and then suspended in the M-9. The OD600 was measured and a dilution was prepared with an OD of 0.01.

Four 1 liter Teflon bottles were prepared with 10 mL Jet A (with 25 mg/L of additive) each. The control sample received 100 mL of M-9 with no *Pseudomonas* while the three replicate sample bottles all received 100 mL of the 0.01 OD *Pseudomonas* in M-9. These bottles were sealed tightly and left in the incubator for eleven days. ODs were measured at day 5 and day 11 and the results are shown in **Table 29**, which indicate that the *Pseudomonas* population was growing well under these conditions.

Table 29. Average optical density values showing the increase in bacterial growth over 11 days

	OD (600 nm)		
	Bottle 1	Bottle 2	Bottle 3
Day 0	0.01	0.01	0.01
Day 5	1.99	1.04	1.93
Day 11	2.40	2.63	2.45

A method was developed to examine BHT in jet fuel. This is not a trivial analysis as it requires a liquid-liquid extraction and analysis using GC-MS with selected ion monitoring. A calibration curve over an appropriate concentration range was developed and is shown in **Figure 32**.

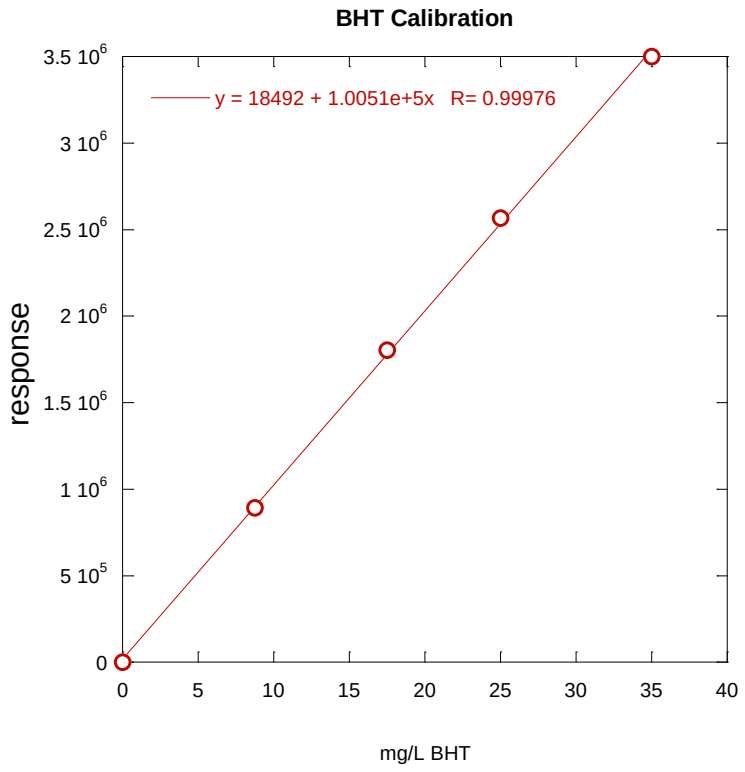


Figure 32. Calibration curve for analysis of BHT in jet fuel.

The curve was applied to the fuels treated with microbes and the results are shown in **Table 30**. There was essentially no difference between the samples exposed to bacteria (R1, R2 and R3) and the control sample (no bacteria). Some small amount of BHT may have been lost during the experiment, but this happened in approximately the same way in each experiment.

Table 30. Concentration of BHT in fuel after exposure to *Pseudomonas aeruginosa.*

sample	Conc. in mg/L
R1	20.4
R2	21.2
R3	21.9
Control	19.0

This experiment showed that *Pseudomonas* did not consume the antioxidant from the fuel. Apparently, the growth occurred in these samples due to the consumption of normal alkanes, which is the typical preference of *Pseudomonas*. This bacteria does not selectively remove antioxidant from fuel.

4.4.2 Corrosion inhibitors/lubricity improvers (CI/LI)

Corrosion inhibitors are required in many fuels; they interact with metal surfaces to slow corrosion. But more importantly, the corrosion inhibitors also provide fuel lubricity enhancement which is also important when fuels are highly hydroprocessed. Hydrotreating also removes the natural lubricants in fuel, and this removal may cause fuel pump failures under extreme conditions. One of the major active components of these additive mixtures is the fatty acid, di-linoleic acid. Bacteria, yeast and fungi can actively degrade fatty acids so marked degradation of these additives was expected. In these experiments, the degradation of the CI/LI "A" was investigated by exposing fuel containing CI/LI to the fuel-degrading-yeast *Yarrowia*. Results showed that CI/LI "A" was degraded during the experiment with *Yarrowia.*

In these experiments, Jet A with CI/LI "B" was added to Jet A at a concentration of 20 mg/L and the neat fuel and sample with CI/LI "B" were measured by LC-MS. The response at the mass for di-linoleic acid (561.48 amu) is shown in **Table 31.**

Table 31 Response for HPLC-MS measurement for CI/LI "A".

Sample	Area Count*
Average Blank	94
Neat Jet A (4658)	7,841
Jet A + CI/LI "A" (20 mg/L)	10,691,762

*Area is of m/z 561.48 signal

CI/LI "B" was added to Jet A fuel at a concentration of 20 mg/L, which is a typical level for jet fuel. *Yarrowia lipolytica* yeast was cultivated for 24 hours in Yeast Peptone Dextrose broth (YPD). A conical tube was centrifuged to pellet out the cultured cells. The YPD broth was decanted and the tube was washed twice with Yeast Nitrogen Broth (YNB) minimal media and then suspended in the YNB. The culture was left in incubator for an additional 24 hours to starve the cells from any remaining carbon source. The OD600 was measured and a dilution was prepared with an OD of 0.01. A significant decrease in concentration of the CI/LI "B" was observed after 14 days (**Table 32**). The microorganism was able to grow to high cell densities in CI/LI mixtures "A" and "B" in Jet A (**Figure 33**). It should not be surprising that the data in **Figure 31** is similar, as the two fuel samples differ only in the type of CI/LI in the fuel. The implication in this data is that bacteria will reduce CI/LI active ingredient concentration in general which may affect fuel lubricity. Additional studies in this area would be useful; decrease in fuel corrosion inhibitor concentration has been shown to negatively affect fuel lubricity.

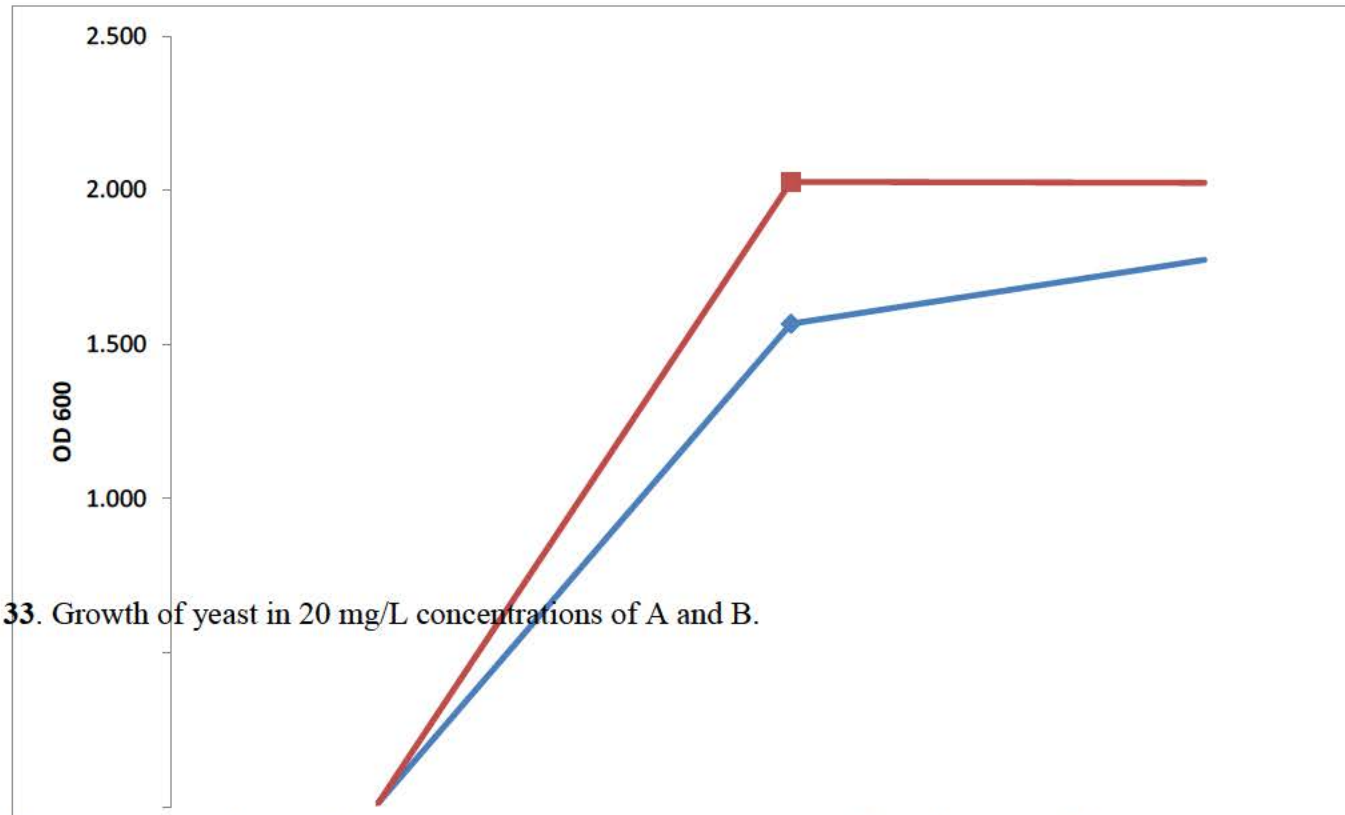


Figure 33. Growth of yeast in 20 mg/L concentrations of A and B.

Table 32. Corrosion inhibitor/lubricity improver "B" concentration in Jet A before and after exposure to *Yarrowia*.

Sample	Area Count	Estimated concentration, mg/L*
Average Blank	401	<0.1
Original Jet A (4658)+CI/LI	8,059,679	20.0
Media exposed Control Jet A (4658)+CI/LI	7,449,995	18.5
Yarrowia-exposed fuel sample R1	1,783,538	4.4
Yarrowia-exposed fuel sample R2	1,909,308	4.7
Yarrowia-exposed fuel sample R3	1,825,077	4.5

*based on the responses at 20 mg/L shown in Table 3.

4.4.3 DiEGME Degradation in Jet A

Fuel system icing inhibitor (diethylene glycol monomethyl ether or DiEGME) is added to jet fuel in concentrations from 0.07 to 0.10 volume %. DiEGME partitions into trace water in the fuel to keep the fuel from freezing. Typical DiEGME in water concentrations in fuel tanks range from 30 to 50 % (v/v/). Bacteria such as *Pseudomonas* have been shown not to survive in concentrations above 5 % (v/v) DiEGME in water. In an attempt to determine if bacteria like *Pseudomonas* will grow in and/or degrade DiEGME in concentrations below that which is toxic to them, *Pseudomonas* was added to DiEGME in water in concentrations ranging between 0.05 and 1 volume % DiEGME. A set of control samples with the same DiEGME concentrations, but without *Pseudomonas* were also prepared. Jet fuel was added above the water layers in a 1:10 jet fuel to water ratio.

The water layers were analyzed for optical density. A portion of each of the water layers was also diluted 1:5 in methanol, and analyzed by gas chromatography- flame ionization detection (GC-FID) in order to quantify the DiEGME concentration. A prominent extra peak was detected in addition to the DiEGME in the samples containing *Pseudomonas*; therefore, the water layer from the 1.0% DiEGME sample was also analyzed by GC-MS. The peak that eluted after the DiEGME was identified as a stable oxygenated metabolite reaction product. It was also quantified by GC-FID, using the calibration generated for quantifying DiEGME.

Two identical sets of 14-day experiments were run in February and May 2014 for repeatability. The bacteria *Pseudomonas* was able to grow effectively in concentrations of up to 1% (v/v) DiEGME (v/v) (**Figure 34**). *Pseudomonas* was also able to degrade 70-80% of the DiEGME when present at lower concentrations (0.05 to 0.20 %), and up to 35% when present at the highest concentration of 1.0 % (**Table 33**). Furthermore, the concentrations of the reaction

product appear to almost be the difference between the initial and the exposed DiEGME concentrations. This result indicated that it may be possible for microbes, especially if rampant bacterial growth occurs in a fuel tank, to reduce the levels of DiEGME to sub-lethal levels in the water phase.

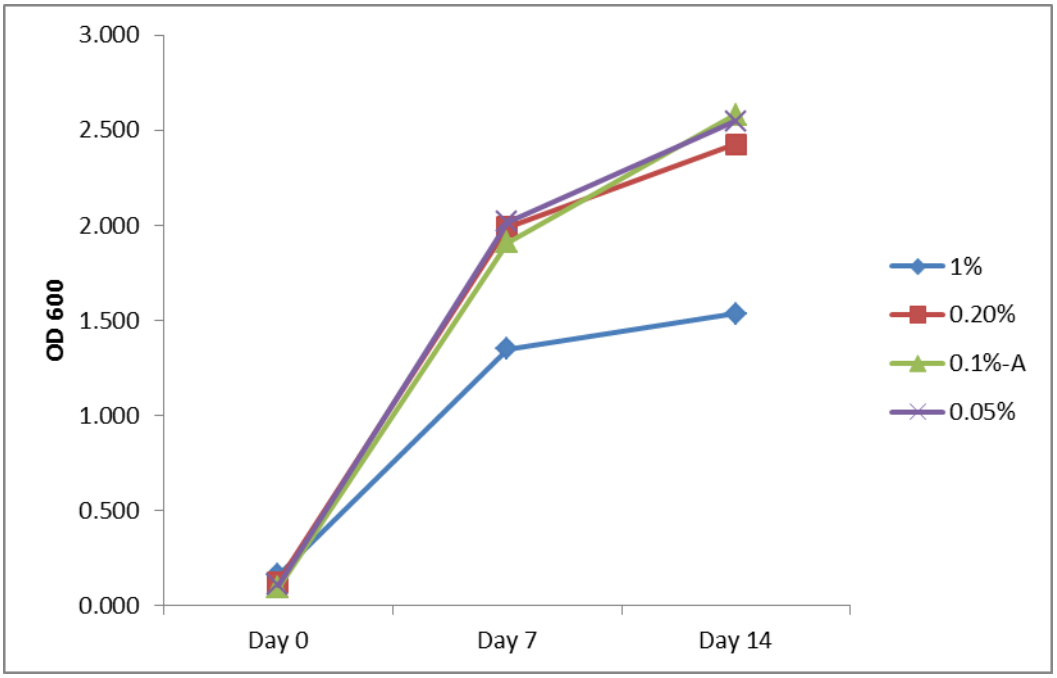


Figure 34. Growth of bacteria in different concentrations of DiEGME.

Table 33. Degradation of DiEGME by *Pseudomonas* in Fuel by Day 14. Mean values for two repetitions (Feb 2014 and May 2014) shown.

Degradation of DiEGME by Pseudomonas in Fuel			
Initial Target DiEGME Concentration (%)	**%DiEGME after 14 Days-Control- Feb-14/May-14**	**%DiEGME after 14 Days-Exposed- Feb-14/May-14**	**% Reaction Product Feb-14/ May-14**
0.050	0.052/0.051	0.017/0.022	0.016/0.019
0.100	0.099/0.097	0.039/0.023	0.038/0.063
0.100	0.094/0.103	0.041/0.060	0.039/0.029
0.100	0.094/0.101	0.040/0.043	0.038/0.046
0.200	0.192/0.200	0.079/0.064	0.083/0.116
1.00	0.915/1.06	0.659/0.725	0.239/0.281

4.5 Effect of FAME Contamination on Jet fuel biodegradation.

More environmentally friendly alternative fuels have been certified for use by the USAF and industry. However, these alternative fuels may potentially be more biodegradable than conventional fuels. Additionally, the possibility of contamination of jet fuel with trace levels of biofuels such as biodiesel or ethanol may further exacerbate biodeterioration. The commercial aviation sector is considering the approval of biodiesel (fatty acid methyl esters / FAMEs) levels in jet fuel at some level between 5 and 100 ppm. While these levels may not seem signficant, in a large jet fuel volumes, this FAME level represents an endless supply of an easy to metabolize high energy carbon source that will promote growth. Here we have tested the effect that trace levels and percentage concentration of FAMEs may have on jet fuel biodegradation. Our results showed that stepwise increases in FAME concentration from 0 to 5, 20 and 100% biodiesel (FAMEs) produced significant increases in cell growth rate and total biomass **(Figure 35)**. More

importantly, we observed that trace FAMEs levels can also increase microbial growth significantly (**Figure 34**). At 100 ppm (0.01%), 800 ppm and 1000ppm (0.1%) FAME microbial growth rate and total biomass dramatically increased over the neat Jet A fuel. Interestingly, when biodiesel is maintained at levels below 50 ppm growth levels are similar to that of neat Jet A. At very low FAMEs level (5ppm) growth appeared lower than in neat Jet A. A likely explanation for this effect could be that the presence of FAMES at low levels is not enough to supply all the energy and carbon required by the cells but this is enough to prevent the activation of the hydrocarbon degradative pathway. We observed that microbes can rapidly carboxylate FAMES, producing fatty acids that are easily metabolized (**Figure 36**). Using GC-MS we observed that exposing biodiesel to bacteria leads to the quick degradation of the Palmitic, Linoleic , Oleic and Stearic methyl esters (**Figure 37).**

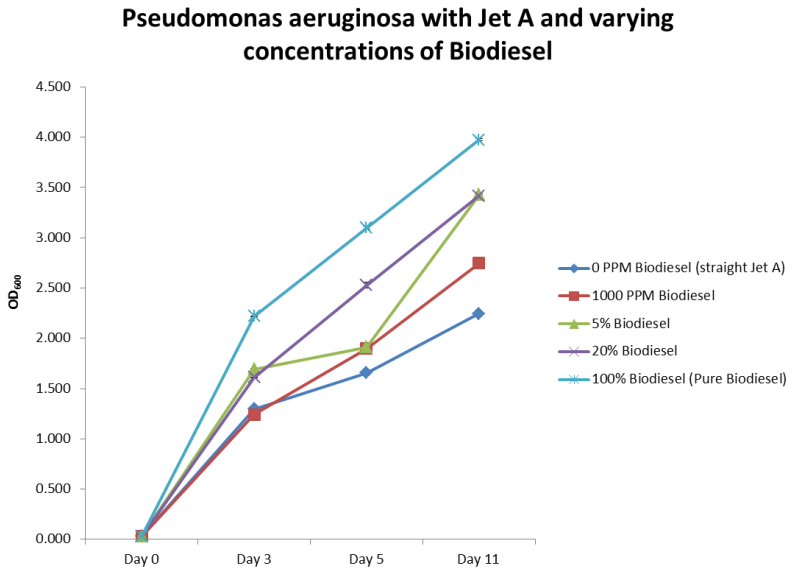


Figure 35. Growth Effect of Percentage Levels of Biodiesel in Jet A

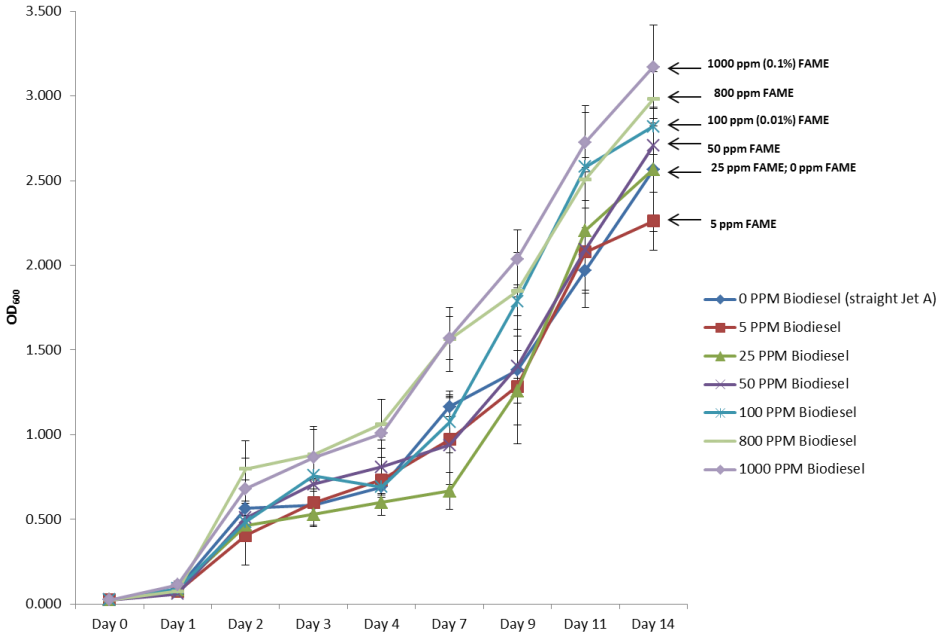


Figure 36. Growth Effect of Low Level Biodiesel in Jet A

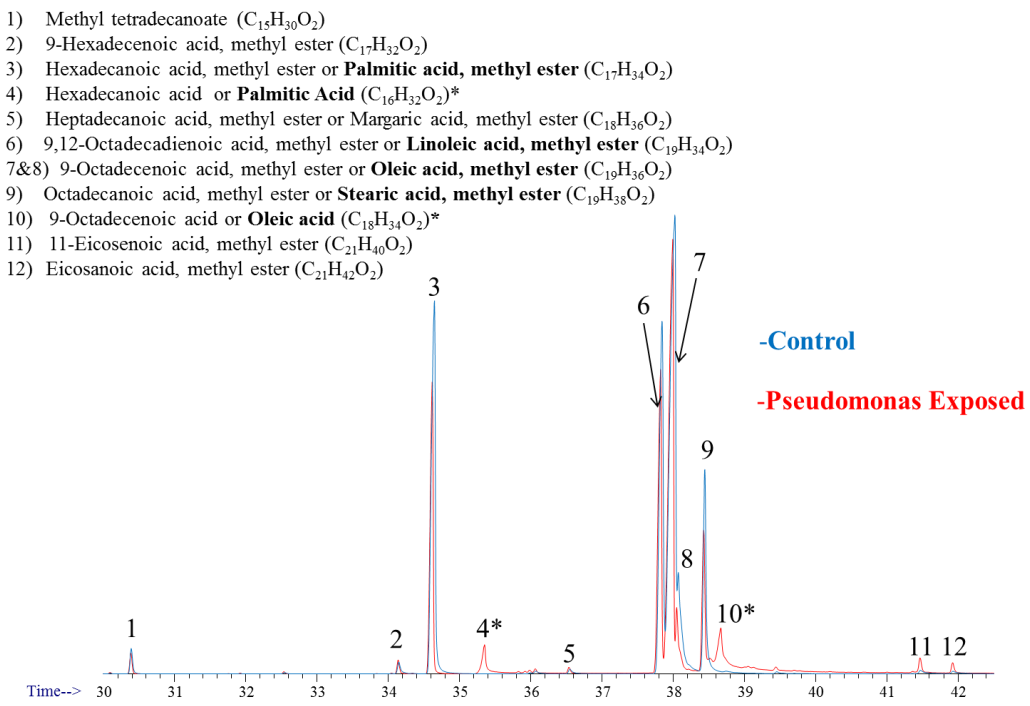


Figure 37. Degradation of FAME Biodiesel by *P. aeruginosa*

4.6 *Acinetobacter venetianus* foam experiments: pumpability, filterabilty

The foam biofilm created by *Acinetobacter* is well documented in the literature. (Baldi, et al., 1999). **Figure 38** shows the cells of *Acinetobacter* surrounding a fuel droplet in order to emulsify the organic fuel. By reacting in this way, these bacteria will form biofilms efficiently as the organisms consume the normal alkanes from the fuel they surround. This creates a thick, foam-like solid which has poor properties for operation in pumps and filters.

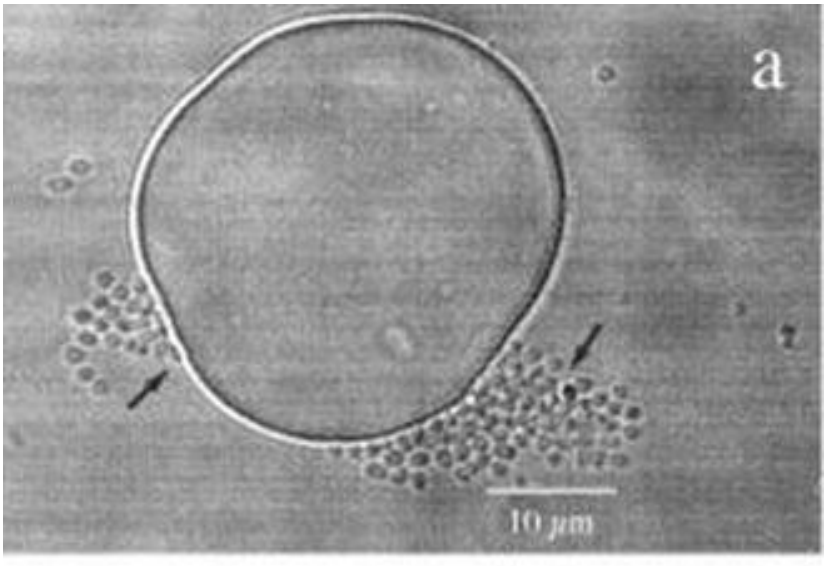


Figure 38. *Acinetobacter* cells (marked by the arrows) surround fuel droplets and later incorporate the fuel into a biofilm emulsion (Baldi, et al., 1999)

Since we have investigated all the tasks proposed in our original proposal, we wanted to examine how this biofilm or emulsified product may affect fuel both in static and flowing situations. We set up a simple flowing tests and filtration devices to show the concentration and extent to which this emulsified product will affect fuel systems.

The examination of the emulsified biofilm material was performed to demonstrate our hypothesis that some amount of this material entrained in a flow system will create significant fuel system problems including filter plugging (as measured by pressure drop), poor fuel flow, disarming of coalescers and similar problems.

A simple ISCO pump flow test was set up in which the pump could be operated a constant flow rate. Pressure can be monitored as the pump is flowing, as there is a pressure transducer in the top of the pump. A flow rate of 1-5 mL per minute was used to investigate the pressure increase of fuel containing small amounts of this foam as it flowed through a small filter, as compared to neat fuel flowing through a filter.

Unfortunately, this experiment was relatively unsuccessful because we could not get the foam material to dissolve into the fuel; the two materials were completely non-homogeneous. The material added to the fuel to be pumped simply went to the side of the container and did not successfully make it to the filter where the pressure was being monitored.

A simpler approach was incorporated where a vacuum filter arrangement was made with a 0.22 micron filter used as per the Jet A filtration time specification test (**Figure 39**). In this test, we simply wanted to determine whether the foam material, if placed on the filter, would increase the filtration time of a specific volume of Jet A. First, 200 mL of fuel was filtered through the system in less than 2 minutes under a low vacuum level. Then, at that same vacuum settting, 2 mL of the foam emulsification was placed on the filter and 200 mL more fuel was filtered through. The fuel was finally passed through the filter after more than 12 minutes of filtering: clearly, the foam is not filterable. Photographs were taken of the polymeric mass trapped on the filter that caused the filter to fail, shown in **Figure 40**.

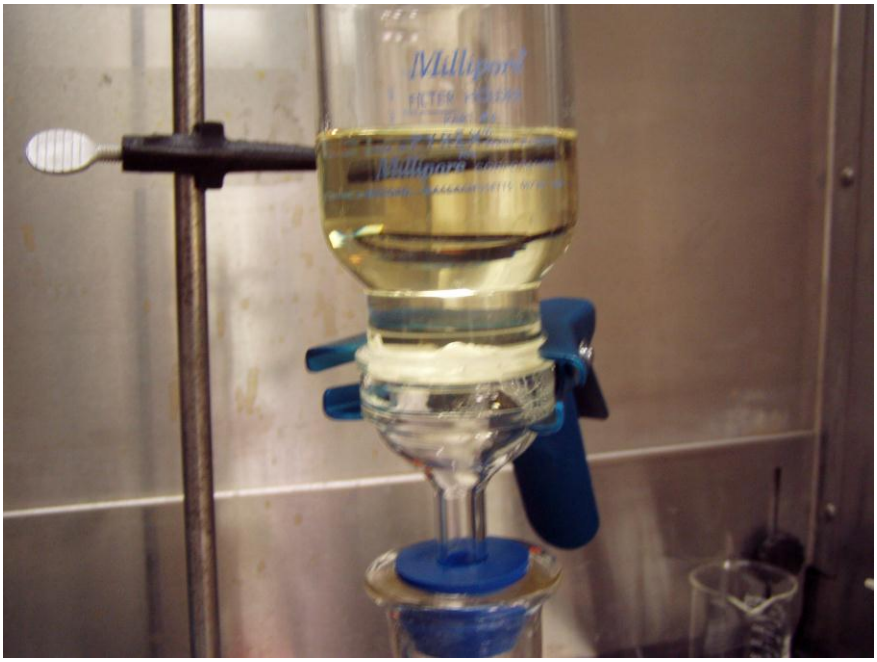


Figure 39. Vacuum filtration as performed in the Jet A filtration time specification test.

Figure 40. (Top) Vacuum fuel filtering is disabled by the emulsion/biofilm material produced by *Acinetobacter venetianus*. (Bottom) Filters were collected from filtering 200 mL of Jet A (left) compared to filtering 200 mL plus 2 mL of the emulsified foam material.

4.7 Fuel lubricity changes due to microbial contamination

Fuel lubricity, as measured by wear scar diameter in a BOCLE test, did not appear to change significantly upon exposure to *Pseudomonas* bacteria. Unfortunately, Jet A does not

contain corrosion inhibitor, and since Jet A was used in the majority of these experiments, the experiments did not show an increase in wear scar. In the case of JP-8 where corrosion inhibitor is part of the standard additive package, we expect that corrosion inhibitor will be consumed by bacteria selectively, as we have shown in **Table 32** above. We believe that this decrease in CI/LI concentration will affect the fuel lubricity. However, we have conducted other experiments (provided in **Tables 18** and **26**) in which the lubricity is improved by exposure to the media (the control sample) and then further improved by exposure to the bacteria. Lubricity is a complicated issue and needs further study. A summary of the fuel lubricity measurements for Jet A experiments is provided as **Table 34.** These experiments show that lubricity improves upon exposure to the aqueous media alone, but also improves due to addition of bacteria. The foam material produced by *Acinetobacter* exposure does not degrade lubricity. The lubricity measurement of Jet A with CI/LI exposed to any bacteria was not performed.

Table 34. Lubricity as measured by BOCLE for Jet A, POSF-4658

Fuel or exposure description	BOCLE measurement, wear scar diameter (mm)
Jet A with no CI/LI, neat, no aqueous exposure	0.71
Jet A with 20 mg/L Unicor J CI/LI, neat, no aqueous exposure	0.57
Jet A with no CI/LI, with 500 ppmv foam emulsion from *Acinetobacter*	0.69
Jet A, no CI/LI, exposed to aqueous media (carboy control sample)	0.57
Jet A, no CI/LI, exposed to *Pseudomonas* and aqueous media (carboy sample)	0.41
Jet A, no CI/LI, exposed to aqueous media in control (DLA-05)	0.57
Jet A, no CI/LI, exposed to aqueous media with *Pseudomonas* (DLA-05)	0.49
Jet A, no CI/LI, exposed to aqueous media in control (DLA-13)	0.57
Jet A, no CI/LI, exposed to aqueous media with *Pseudomonas* (DLA-13)	0.54

5. CONCLUSIONS

The microbial degradation of jet fuel, diesel fuels and other hydrocarbons occurs readily in fuel-water environments. The microbes studied in this project include *Pseudomonas aeruginosa, Acinetobacter venetianus, Marinobacter hydrocarbonoclasticus and Yarrowia lipolytica* because of their common occurrence in fuel systems. These microorganisms each have as specific group of compounds which they most prefer to consume: *Pseudomonas, Acinetobacter* and *Yarrowia* prefer a wide range of n-alkanes from C10 to C22 and *Marinobacter* prefer lighter normal and branched alkanes, and some aromatic compounds between C8 and C10. Small sample vial testing determined these tendencies.

Experiments were also conducted to create larger amounts of exposed fuel which could then be submitted for specification testing, to determine if microbial degradation would affect the overall properties of fuel as determine by specification tests. Because these bacteria are mainly n-alkane consumers, the only significant changes observed were low temperature properties such as freeze point, pour point and cloud point, all of which improved because of the selective consumption of heavy alkanes. However, bulk properties (density, distillation range, aromatic content, heat of combustion, etc.,) were not greatly affected. Other properties such as Total Acid Number, sulfur content, flash point, smoke point, lubricity, etc., were not affected by exposure to bacteria under the conditions that were tested.

Additives tested such as DiEGME and Corrosion Inhibitor/Lubricity Improver (CI/LI) were consumed, apparently, by some bacteria. In the case of DiEGME, this additive was

designed to be soluble in water, and bacteria will reduce its concentration in the water. As tempertures become lower, however, more DiEGME will partition into the water and create a high enough concentration to be toxic to the bacteria. The effect of CI/LI reduction may create a variety of problems on board an aircraft.

While some concerns about additive reduction and small changes in fuel composition are important considerations in the exposure of fuel to microbes, the formation of biofilm is by far the major concern. Biofilms are viscous, capable of failing specification tests relating to filtration time, thermal stability, storage stability, existent gum, and other tests. We demonstrated briefly that the formation of this biofilm will actually plug filters which will create problems. Future work in this area should focus on this biofilm and problems that this could cause operationally, including fuel gauging, pumping and other related problems.

6. ACKNOWLEDGEMENTS

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